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THE ROLE OF STAT AND THE JAK/STAT PATHWAY IN MEDIATING THE EFFECTS OF INTERLEUKIN-6 ON STAR EXPRESSION

By

Janae Strickland

A thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Physiology and Developmental Biology

Brigham Young University

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BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

Janae Strickland

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the thesis of Janae Strickland in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

THE ROLE OF STAT AND THE JAK/STAT PATHWAY IN MEDIATING THE EFFECTS OF INTERLEUKIN-6 ON STAR EXPRESSION

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Cortisol, a hormone produced by the adrenal gland, is responsible for many regulatory functions in the body. Cortisol release is mediated by adrenocorticotrophic hormone, or ACTH, through the hypothalamus-pituitary-adrenal or HPA axis. This HPA axis is the major release pathway used during acute stress, during which the levels of ACTH parallel those of cortisol. However, in states of chronic stress, the level of ACTH drops dramatically, while cortisol remains high. This study focuses on the pathway of cortisol release during these chronic stress states, specifically examining the role of IL-6 with respect to STATs and the Jak/STAT pathway. It has been shown that IL-6 increases cortisol levels, and that IL-6 utilizes the Jak/STAT pathway. Also, the steroidogenic acute regulatory (StAR) promoter contains multiple STAT binding sites. Thus, STATs could be mediating the effects of IL-6 in the chronic release of cortisol by inducing expression of StAR.

Experiments were performed to identify whether IL-6 has a direct effect on StAR promoter activity, StAR mRNA and StAR protein levels. Electromobility Shift Assays (EMSA) were performed to show that STATs bind to the full STAT site within

the StAR promoter region. Various experiments were also carried out in the presence of IL-6 alone or, congruently with either a Jak (AG490) or STAT3 (Piceatannol) inhibitor, to show the effects of STATs and the Jak/STAT pathway on StAR. Luciferase assays were performed in order to observe the effects on induction of the StAR promoter. RT-PCR and western blots were also performed to observe the effect of Jak/STAT inhibition on both StAR mRNA levels and StAR protein levels. These experiments showed a marked decrease in the IL-6-stimulated StAR promoter activity, mRNA and protein expression when treated with wither Jak or STAT inhibitor. Therefore, IL-6 regulates expression of StAR through utilization of the Jak/STAT pathway; which phosphorylates and subsequently dimerizes STAT, allowing STAT to translocate to the nucleus and bind to the StAR promoter, thus increasing StAR expression and thereby inducing synthesis of cortisol.

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Thanks should also be given to my parents for all of their support and for always believing in me. As well as to my dog Max, who made life just a little more bearable each day.

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INTRODUCTION

The Adrenal Gland

The adrenal gland is an important endocrine organ, located directly on top of the kidney, and is triangular in shape. The adrenal gland is comprised of two basic layers, the outer adrenal cortex and the inner adrenal medulla. The adrenal cortex can be subdivided into three distinct layers: the outer zona glomerulosa, the middle zona fasciculata and the inner zona reticularis. All of these distinct layers release steroid hormones, which are crucial to normal physiological functions. The zona glomerulosa is responsible for secretion of aldosterone, whereas the zona fasciculata releases cortisol, the zona reticularis releases dehydroepiandrosterone (DHEA), and the adrenal medulla releases epinephrine. The steroid hormones, aldosterone, DHEA and cortisol, are vital to the maintenance of the human body and in carrying out normal endocrinological functions necessary for life.

Cortisol and Steroid Hormone Production

Steroid production, namely cortisol, plays a major role in the onset and control of various diseases. Cortisol plays roles in metabolism and blood sugar maintenance, blood pressure regulation, bone formation, protein metabolism, and is crucial in inflammatory and immune responses. Cortisol is released from the zona fasiculata of the adrenal cortex. When cortisol is released, the concentration of plasma cholesterol, glucose and fatty acids likewise increases. Cortisol also inhibits the inflammatory response in many tissues, as well as inhibits cell-mediated immunity, making it very difficult for the body to fight infection as efficiently. Cortisol release, in acute stress, is

stimulated through the classic hypothalamic-pituitary-adrenal (HPA) axis. In this model, corticotrophin releasing hormone (CRH) is released from the hypothalamus, which in turn stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH then acts on the adrenal gland to induce the release of cortisol. Since, under acute stress, the correlation between plasma ACTH levels and plasma cortisol levels is strong, it can be deduced that the HPA axis is the release mechanism used primarily under such acute conditions. However, this relationship greatly deteriorates under chronic stress, specifically, while cortisol levels remain high ACTH levels drop, no longer running parallel to those of cortisol. Consequently, this fact demonstrates that other mechanisms must be responsible for chronic cortisol release.

Interleukin-6, An Important Cytokine

Cytokines, which are small peptides or proteins that act as signaling molecules for cellular interactions, play important roles in the acute immunological and inflammatory responses within the body. During these responses, cytokine production and secretion is increased. Interleukin-6 (IL-6), a cytokine, is synthesized and released from the adrenal gland. Not only is IL-6 synthesized in the adrenal gland, it has also been found to modify the function of the adrenal gland (8). It has previously been discovered that IL-6 stimulates cortisol release from the bovine adrenal cell in a time-dependent manor (26). Previous findings also demonstrated that IL-6 is a major contributor of chronic corticosterone (a steroid closely related to cortisol) release from the adrenal glands of mice during inflammatory or immunological responses (19). This

information led many to wonder how IL-6 stimulated such a response. During immune responses, it is widely accepted that IL-6 utilizes the Jak/STAT pathway to carry out many important functions, such as stimulation of the liver to produce acute-phase proteins, stimulation of the production of B-lymphocytes and increases in neutrophil production. IL-6 is known to utilize the Jak/STAT pathway within immune cells but it is unclear whether IL-6 utilizes this same pathway when mediating its effects within the adrenal gland and steroidogenesis. Therefore, if IL-6 utilizes this pathway for its immunological functions, then IL-6 may also be inducing sterodiogenic acute regulatory (StAR) expression and therefore cortisol release through use of the Jak/STAT pathway within the adrenal gland.

JAK/STAT Pathway

As previously stated, IL-6 acts through the Jak/STAT pathway to carry out its necessary functions within immune cells and other tissues; therefore, understanding of this pathway is crucial to understanding the chronic release mechanism of cortisol release from the adrenal. Figure 1 illustrates the current model of the IL-6/gp130/Jak-STAT pathway, which is found within many cells of the immune system. First, IL-6 binds to the cell-surface receptor, gp80. Binding of IL-6 induces dimerization of two gp130 molecules; this dimerization induces the phosphorylation of associated Janus Kinase 2 (Jak2), by tyrosine heads (7). Jak, once phosphorylated, is activated. Upon activation of Jak, the cytoplasmic part of gp130 is then phosphorylated, which in turn creates docking sites for signal transducers and activators of transcription (STAT), specifically STAT1 and STAT3. The STATs then bind to these docking sites and

become, themselves, phosphorylated. Once phosphorylated the STATs are released from the receptor and form homo- or hetero-dimers, which are then able to translocate into the nucleus. Once inside the nucleus, STAT may bind to specific sites on the important genes that regulate steroidogenesis (7). It has been demonstrated that IL-6 acts on this pathway by facilitating phosphorylation of both Jak and STATs. For IL-6 to induce cortisol production and release, STAT must bind to specific genes which regulate steroidogenesis, thereby increasing transcription of these necessary genes and increasing cortisol production and release.

Steroidogenic Acute Regulatory Protein (StAR)

Steroid hormone production and expression is a slow process, biologically speaking, in that it involves transcription of specific genes within the nucleus of a cell. Steroidogenic acute regulatory (StAR) gene has been shown to have increased transcriptional activity during steroidogenesis in bovine adrenal cells (26). Transcription of the StAR gene, a crucial gene in the rate-limiting step of steroidogenesis, is induced by IL-6, steroidogenic factor 1 (SF-1) and various members of the activator protein-1 (AP-1) system. StAR protein is responsible for the transportation of cholesterol from the outer to the inner membrane of the mitochondria, and therefore, is crucial to steroidogenesis, since all steroid hormones use cholesterol as their precursor (21). The movement of cholesterol into the inner membrane of the mitochondria makes it readily available to form pregnenolone, which is the first step in steroid hormone formation. Pregnenolone is formed by the cleavage of cholesterol through the action of the enzyme P450 side chain cleavage (P450scc). Through various enzymatic cleavages of

pregnenolone, all previously mentioned steroid hormones (Aldosterone, DHEA and Cortisol) can be formed (see Figure 2).

The mechanism by which StAR imports cholesterol into the mitochondria is thought to occur by creation of a hydrophobic tunnel, by means of the StART domain of StAR. This StART domain undergoes a conformational change and then traverses the outer membrane and appears at the surface of the intermembrane space, acting as a channel through which cholesterol can enter at one end and exit at the other. Eventually, the entire StAR molecule enters the matrix and is no longer active in further cholesterol transfer. Thus, the hydrophobic core is the means for cholesterol transfer only for the length of time that is required for StAR to be entirely imported into the matrix (27, 23).

In previous studies, utilizing bovine adrenal fragments and the human adrenal tumor cell line H295R, it has been shown that as IL-6 increases the level of StAR mRNA expression increases (25). The levels of StAR mRNA expression should therefore correlate with StAR protein expression and therefore the amount of steroid production taking place within the tissue. Also, the StAR promoter has binding sites for STATs therefore these proteins may also be involved in the regulation of StAR (Fig 3). Experimentation was done to determine the mechanism through which IL-6 increases StAR mRNA expression. Our goal was to answer the following questions: 1) does STAT bind to the StAR promoter and consequently induce transcription of StAR and 2) is the binding of STAT specific and necessary for StAR expression and therefore proper steroidogenesis?

The purpose of this study was to better elucidate the pathway of chronic cortisol release mediated through IL-6. First, human adrenal cells (H295R cells) were treated with either different dosages of IL-6 (IL-6 dose-response) for 40 minutes or with a standard dose of IL-6 for variable times (IL-6 time course). These experiments showed that IL-6 affected StAR expression in both a time-dependent and dose-dependent manner. Dose and time-dependent experiments were performed in order to mimic the regulatory mechanisms within the human body which are subject to time and dose in order to control vital functions and responses. Also, through the use of specific inhibitors of Jak and STAT, we were able to show a direct involvement of STAT with the StAR promoter and demonstrate changes in StAR expression upon inhibition of Jak or STAT in both the presence and absence of IL-6 stimulation. Electromobility shift assays (EMSA) showed that upon stimulation with IL-6 alone a band shift occurred, suggesting that IL-6 induced the binding of STATs directly to the StAR promoter region. Upon incubation with either inhibitor there was no shifted band observed, as well as with simultaneous treatment of IL-6 with either inhibitor. These findings suggest that IL-6 utilizes the Jak/STAT pathway to induce StAR expression, as the shift and thus binding of STATs to the StAR promoter were only observed upon treatment with IL-6. Reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot experiments showed correlated results to those of the EMSA, specifically that IL-6 increased both StAR protein levels and mRNA levels and exposure to Jak/STAT pathway inhibitors markedly decreased both IL-6-induced StAR mRNA and protein levels. Luciferase studies showed similar responses, IL-6 greatly induced StAR promoter activity, while Jak/STAT inhibition decreased IL-6-induced promoter activity.

Therefore, we found that the effects of IL-6 on StAR expression, and thus cortisol release are, at least, directly mediated through the binding of STATs to the StAR promoter. Other pathways may be present, wherein STATs binds to other StAR regulatory genes, such as dosage-sensitive sex reversal-adrenal hypoplasia gene on the X chromosome, gene 1 (DAX-1), steroidogenic factor 1 (SF-1) or components of the activating protein (AP-1) system, thereby indirectly influencing the expression of StAR and thus cortisol production and release. Also, STATs may bind to the genes that regulate the expression of necessary steroidogenic enzymes, such as CYP11A1, CYP11B1, CYP17A1 and CYP21A1, utilized in the pathway of cortisol synthesis, thereby stimulating cortisol release in a mechanism independent of StAR expression. However, further experimentation is needed in order to better elucidate the role these other pathways may have on cortisol production through mediation by IL-6. Our findings, however, point to a direct involvement of STATs with the StAR promoter, thus helping to drive IL-6-dependent release and synthesis of cortisol.

MATERIALS AND METHODS

Materials

Recombinant mouse IL-6 was a gift from J. Van Snick (Ludwig Institute, Brussels, Belgium). The IL-6 was diluted using (DMEM) Dulbecco's Modified Essential Medium (Gibco, Carlsbad, CA) to a working stock concentration of 100 ng/ml and stored in 10 µl aliquots at -94 ˚C.

Tissue Culture

H295R cells (American Type Culture Collection, Manassas, VA) were cultured at 37ºC with 5% CO_2 in supplemented DMEM/F-12 (Gibco). Supplementation was with 80 IU/ml penicillin, 80 µg/ml streptomycin (Gibco), 0.1% ITS and 5% Nu Serum (HyClone, Logan, UT).

Luciferase Reporter Gene Assay

Transfection – H295R cells were transfected with 4 µg of the luciferase gene containing plasmid (pGL2), or 12 µg of a 6456 base pair, pGL2 plasmid modified to contain the StAR promoter region (-858bp), and 7 µg of a β-galactosidase expression plasmid used here as an internal control for transfection efficiency.

Lipofectamine-PLUS Reagents (Invitrogen, Carlsbad, CA) were used to transfect the cells following manufacturer's instructions. Briefly, each well of a 12-well plate was seeded with 1.25×10^5 cells and incubated 18-24 hours with supplemented DMEM (see Tissue Culture). A solution of 0.6% PLUS reagent and plasmid DNA was incubated in DMEM/F-12 (Gibco) + ITS (supplemented with only ITS), for each

plasmid (see above for amounts), for 15 minutes at room temperature. Simultaneously, a solution of 0.6% Lipofectamine was also incubated at room temperature. Lipofectamine solution was then combined with PLUS/plasmid solution and subsequently incubated at room temperature for 15 minutes to create the transfection solution. While the transfection solution was incubating, 400µl of DMEM/F-12 + ITS was added to each well. Following incubation, 100µl of transfection mix was added to each well and incubated 5-6 hours. Transfection mixture was removed and 1ml of 2X Serum DMEM/F-12 media (10% Nu Serum, without ITS or antibiotics) was added to each well and cells were incubated 16 – 24 hours. This was followed by a 15 – 30 minute incubation in fresh DMEM/F-12 + ITS (without supplementary serum and antibiotics). Cells were then treated, with specific treatments and likewise with the following controls; cells transfected with the empty basic plasmid and treated with serum-free (SF) DMEM and also cells transfected with both StAR and β-gal plasmids and treated with SF-DMEM. After treatment, cells were subsequently rinsed with cold phosphate buffered saline (PBS). The PBS was removed and 75 µl of Cell Extraction solution *(83mM* K_2HPO_4*, 16.63mM* KH_2PO_4*)* was added to each well. Cells were harvested by scraping, and cell debris pelleted by a 10 minute centrifugation at 14,000 rpm.

Luciferase Assay – β-galactosidase production was measured using the Galacto-Light Plus system (Tropix/Applied Biosystems, Foster City, CA) according to manufacture's instruction. Briefly, 100 µl reaction buffer (substrate plus diluent) was added to cell extract (2 µl). After 30 minutes of incubation, 100 µl of accelerator (Tropix) was added to terminate the enzyme activity and trigger light emission

measured by the luminometer (TD-20/20 Turner Designs, Sunnyvale, CA). Luciferase production in transfected cells was measured using the Luciferase Assay System (Promega, Madison, WI). Luciferase assay reagent (100 µl) containing luciferin substrate was added to cell extract (20 µl) and light produced from the luciferase reaction was measured by the luminometer. Results were graphed using Microsoft Excel, including standard errors, by calculating the ratio of Luciferase/ β-galactosidase light production or relative light units (RLU). Ratios were then normalized to serum free DMEM levels, thus final expression was in terms of fold RLU expression.

Reverse Transcriptase-Polymerase Chain Reaction

mRNA extraction – IL-6 time-course experiments were carried out by treating H295R cells with 5 ml of serum-free DMEM containing either 10 or 50 pg/ml of IL-6 and incubating at 37˚C and 5% CO_2 over various amount of time (ranging from 10 to 270 minutes). Dose-response experiments were performed by incubation with various levels of IL-6 (0.1pg/ml to 100pg/ml), for 40 to 60 minutes. mRNA was extracted from H295R cells after treatment, using TriZol reagent (Gibco) according to manufacturer's instructions. The mRNA pellets were dissolved in 50 µl of nuclease-free water (NFW), concentration of each sample was determined by spectrophotometry and finally diluted in order to obtain working concentrations between 1000 and 2000 µg/ml.

RTPCR – 2 µg mRNA was added to 7.4 µl of a mixture containing, 10x PCR buffer, dNTP, 100mM dithiothreitol (DTT), Random Decamer, RNase Out (Gibco) and Superscript II Reverse Transcriptase (Invitrogen). Nuclease-free water was added to bring the total volume of the reaction mixture to 20 µl. Reactions were reverse

transcribed at 42˚C for 60 minutes, followed by rapid cooling to 4˚C in a programmable thermocylcyer (Perkin-Elmer Series 9600, Wellesley, MA).

PCR reactions were run by adding 2 µl of each RT to 48 µl of a grand mixture containing, 37.375 µl NFW, 5 µl 10X PCR buffer, 1 µl dNTP, and 0.6 µl JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO) and then 1 µl each of the complementary primer set (a separate grand mixture was made for each primer set, StAR and 18s). Human StAR and 18s primers were custom ordered (Invitrogen) as follows, forward StAR primer: *5'-cccatggagaggctctatgaag-3'* and reverse StAR primer: *5'-gaccttgatctccttgacattgg-3'* also 18s sense primer: *5'-cctttaacgaggatccattgga-3'*, and the 18s antisense primer: *5'-ttgctgcagttaaaaagctcgtag-3'* each of which were diluted to 0.1nmole/µl. Complementary 18s primer strands were used as experimental procedure control as well as a comparative strand of efficiency. These 18s primers were diluted with competimers in a 1:1 ratio before being added to the grand mixture to ensure 18s cDNA amplification occurred in the linear range.

18s and StAR PCR reaction mixtures were heat-cycled in the programmable thermocycler as follows: pre-incubation for 8 min at 95ºC, then 50 cycles of 5 s at 95ºC, 5 s at 62ºC, then 13 s at 72ºC, final elongation period of 5 minutes at 72ºC, followed by rapid cooling to 4ºC. The number of cycles ran was chosen due to the fact that StAR mRNA is extremely unstable and easily broken down (6).

Anaylsis of PCR products – Each PCR sample was mixed with 2 µl of gel loading dye and then loaded into a 0.5% Agarose gel containing ethidium bromide. The cDNA samples were run through the gel at 80V for approximately 30 minutes. The ethidium bromide stained samples were visualized under UV illumination and the

images were recorded by use of the Fluorchem 8900 Imaging System (Alpha Innotech, San Leandro, CA). Finally, quantification of the cDNA bands was performed using the SpotDenso densitometer program (AlphaEase FC software, Alpha Innotech). StAR to 18s ratios were calculated, fold mRNA expression from that of serum-free DMEM levels were then plotted, with standard error bars, using Microsoft Excel.

Western Blot

Protein Extract – Protein was obtained from H295R cells after appropriate treatment. Plates were washed with 1X PBS, then exposed to 0.6mL of RIPA buffer *(1% Nonidet P-40, 12mM Sodium Deoxycholate, 3.5mM Sodium dodecyl sulfate (SDS), dissolved in 1X PBS; plus the following inhibitors, 1% PMSF (phenylmethyl sulfonyl fluride, in isopropanol), 3% Aprotinin, and 1mM of Sodium orthovanadate added at time of use)*, for 15 minutes on ice. Cells were scraped, then centrifuged, and lysates collected and stored at -20˚C. Protein content was determined through the Bradford Assay.

Western Blot – In brief, samples were prepared first by diluting protein extracts in sample buffer. Samples were run on a 10% precast Tris-HCl Gel (BioRad, Hercules, CA) using 1X Tris-glycine-SDS running buffer at 200V for 30 minutes. Protein was transferred to either nitrocellulose membrane (BioRad) or Biodyne B nylon membranes (Pierce, Rockford, IL) using 100V for 30 minutes. Membranes were blocked with 5%Milk-PBST solution, washed with two cycles each of 1X PBST (for 10 minutes each) followed by 1X PBS (for 5 minutes each), and then incubated overnight at 4˚C while shaking with 1˚ StAR Antibody (SantaCruz, Santa Cruz, CA), which was diluted

1:500 in a 5% milk-PBST solution. A secondary Goat-Anti-Rabbit (SantaCruz) antibody, diluted 1 in 700 was then added and then again washed with two cycles each of 1X PBST (for 10 minutes each) followed by 1X PBS (for 5 minutes each). Protein bands were detected with a luminol/peroxidase reagent (SantaCruz) and exposed to X-Ray film. Protein levels, in terms of fold protein expression were normalized to serum-free DMEM levels, were quantified using FluorChem software and plotted, including standard error bars, with Microsoft Excel. It should be noted that protein levels were calculated to adjust for the background effect; specifically, the background levels surrounding each specific protein band were calculated and subtracted from the protein levels observed. Fold protein expression was determined from the background-adjusted levels.

Electrophoretic Mobility Shift Assay (EMSA)

DNA Probe/Custom Primer Synthesis **–** The complete sequence of the human StAR promoter was identified using the National Center for Biotechnology Information (NCBI) website. Upon close examination, four possible STAT binding sites were identified, with only one containing a full binding site (Fig. 3). STATs sites are either full sites, meaning STATs bind to both sense and antisense strands of the DNA site, or half sites which only bind to one strand or the other. The DNA primer was chosen so as to contain the full STAT1 and STAT3 binding site. The complementary primers were then ordered through Invitrogen, using the following sequence: (-1034bp with a total length of 30bp, outlined by a red box in Figure 3), *5'-actgtgtgccttcatctaagctgcccctgc-3'*. These custom primers were used as the DNA probe in the EMSA experiments.

Complementary single-stranded DNA primers were annealed by incubating at 90°C for 20 minutes and then slowly cooled to RT and stored at -20°C until used.

Nuclear Extracts – Nuclear extracts were obtained from H295R cells after treatment. In brief, cells were first rinsed with cold PBS and then scraped and centrifuged to pellet cells. Pellets were resuspended in 1ml of cold Membrane Lysis Buffer *(10mM Hepes pH 8.0, 1.5mM $MgCl_2$, 10mM KCl and 1.0mM DTT; Triton X was added at use in 1000:1 dilution)* and incubated 15 minutes on ice. Following the incubation on ice, 100 µl of 10% Igepal-CA630 solution was added, followed by a quick vortex. Cells were pelleted by centrifugation for 3 minutes at 14,000 rpm. Pellets were resuspended in 175 µl of cold Nuclear Envelope Lysis Buffer *(20mM Hepes pH 8.0, 1.5mM $MgCl_2$, 25% glycerol, 420mM NaCl, 0.2mM ethylenediaminetetraacetic acid (EDTA), and 1.0mM DTT; again Triton X added at 1000:1 dilution at use),* and vortexed briefly. Cells were rotated for 30 minutes at 4ºC, then centrifuged and supernatant collected and analyzed for protein content by Bradford Assay.

EMSA – EMSAs were performed in order to observe DNA-protein interactions, specifically STATs with the StAR promoter region. EMSAs were carried out using custom primers of the full STAT1/3 binding site as DNA probes (Figure 3), in order to show binding of STATs to the StAR promoter. EMSAs were carried out as follows. In brief, ssDNA probes were annealed to form dsDNA probe, by incubating at 90˚C for 20 minutes, and cooled to RT slowly. DNA-Protein binding reactions were performed using custom annealed dsDNA STAT probes (0.1pg each individual strand) and nuclear extracts (1µg) from H295R cells. A mixture of the following was added to each binding reaction, 2µl of 10X binding buffer (Pierce, LightShift™10X Binding Buffer),

1µl 1% Igepal CA630 solution (Sigma-Aldrich), and 1µg Poly dI·dC (GE Healthcare Bio-Sciences, Piscataway, NJ). The samples were then incubated at room temperature (RT) for 20 minutes. Binding reactions were then loaded on a 5% TBE Gel (BioRad) and run at 100mV for 150 minutes, using 0.5X TBE Running Buffer. The DNA/protein complexes on the gel were then transferred to nylon membrane (BioDyne B, Pierce) utilizing the Mini-Transblot system (BioRad), at ~10˚C and 380mA for 30 minutes, with 0.5X TBE as the transfer buffer. DNA was then cross-linked to the membranes using a UV transilluminator at 302nm for 20 minutes. The membranes were then exposed to blocking solution, washed and incubated overnight at 4˚C, while shaking, with STAT-3 primary antibody, diluted 1:500 using 5% Powedered milk-PBST (Santa Cruz). A secondary anti-goat-anti-rabbit antibody, diluted 1:700 with 5% milk-PBST solution, was then added and detection was performed by exposing the membranes to a luminol/stable peroxidase detection solution. Exposure of films to membrane varied from 1 to 12 minutes.

RESULTS

IL-6 induced expression of StAR in a Dose-Dependent Manner

StAR promoter activity was induced by IL-6 in a dose-dependent manner – Luciferase assays were performed using pGL2-luc plasmid, containing the StAR promoter and a β-galactosidase plasmid as control. H295R cells were transfected with plasmids, and subsequently treated with increasing amounts of IL-6 (varying from 1pg/ml to 100pg/ml). Relative light units were determined for each sample, by dividing light production of the Luciferin-StAR plasmid by β-gal light production. As the dose of IL-6 increased, fold relative light unit expression also increased. Thus, StAR promoter activity increased in a dose-dependent manner; meaning, as IL-6 increased StAR promoter activity increased thereby, presumably inducing a greater transcriptional activity of the StAR gene. The peak activity was detected as soon as 5g/ml IL-6 with a 1.4-fold RLU increase observed (Figure 4) after which fold expression began to plateau.

The effects of IL-6 on the levels of StAR mRNA were dose-dependent – An IL-6 dose-dependency was also observed with StAR mRNA levels. RT-PCR was performed using human StAR sense and antisense primers, along with human 18s primers to determine the StAR mRNA levels, after 50 cycles of DNA amplification, within H295R cells. Cells were treated with increasing amounts of IL-6 (from 0.1pg/ml to 100pg/ml) for four hours and mRNA was extracted using TriZol according to manufacturer protocol (Gibco). Figure 5a shows an increased density of StAR bands, relative to 18s, upon increased dosages of IL-6. As with StAR promoter activity, the peak of fold StAR mRNA expression (3.2-fold increase) occurred at 5pg/ml IL-6, followed by a plateau through the 50pg/ml IL-6 dosage, at which mRNA expression

decreased at 100pg/ml (Figure 5b). Calculations of fold mRNA expression are more difficult to measure due to the high instability of StAR mRNA (6).

IL-6 acted in a dose-dependent manner to induce StAR protein expression – IL-6 also increased StAR protein expression in a dose-dependent manner. Specifically, western blots were performed using human adrenal tumor cells (H295R). Cells were treated with a range of IL-6 dosages, 1pg/ml to 100pg/ml (Figures 6a and 6b). As with StAR promoter activity and mRNA levels, protein levels also increased upon treatment with each higher dose of IL-6. Maximum expression was seen upon treatment with 100pg/ml, which increased StAR protein levels 1.81-fold. It is important to note that the fold protein expression increased 1.78-fold upon exposure to 1pg/ml and peaked again upon treatment with 100pg/ml IL-6, with a dip occurring at the 10pg/ml dose.

IL-6 induced expression of StAR in a Time-Dependent Manner

IL-6 acted in a time-dependent manner to induce StAR promoter activity – Luciferase assays were performed using pGL2-luc plasmid, containing the StAR promoter and a β-gal plasmid as control. Cells were transfected and subsequently treated with 10pg/ml IL-6 for increasing amounts of time: 0, 10, 30, 60, 90, 120, and 240 minutes. Relative light units were determined for each cell lysate. From the results, we determined that as time of treatment with IL-6 increased, fold relative light unit expression also increased. Thus, StAR promoter activity increased and consequently transcriptional activity of the StAR gene increased as time of treatment increased, therefore displaying a time-dependent curve (Figure 7). StAR promoter activity demonstrated a bi-phasic time effect, with the maximal peaks observed first at

30 minutes post treatment, with a 1.52-fold RLU expression seen above basal levels followed by a peak at 90 minutes of treatment with IL-6, in which a 1.53-fold increase was observed.

StAR mRNA was induced by IL-6 in a time-dependent manner **–** An IL-6 dose-dependency was observed with StAR mRNA levels. RT-PCR was performed using human StAR sense and antisense primers, along with human 18s primers to determine the StAR mRNA levels within H295R cells. Cells were treated for increasing amounts of time (0, 20, 40, 60 and 80 minutes) with 10pg/ml of IL-6. In order to run a subsequent RT-PCR, mRNA was then extracted and quantified. An increase in the intensity of the fold mRNA expression of StAR relative to 18s was observed upon increased time of treatment (Figure 8a). The 3.0-fold mRNA expression peak occurred upon treatment for 20 minutes, after which the levels plateaued (Figure 8b). Fold mRNA expression measurements were difficult to obtain due to the destabilizing factors acting within or on StAR mRNA (6).

The effects of IL-6 on the levels of StAR protein were time-dependent **–** IL-6 also showed an increase in StAR protein expression in a time-dependent manner. H295R cells were treated for various lengths of time (0-240 minutes) with 50pg/ml of IL-6, and then subsequent quantification and western blots were performed using StAR antibodies (SantaCruz) (Figures 9a). As with the StAR promoter activity and mRNA levels, an increase in fold protein expression was observed as treatment time increased. A general upward trend was seen as time increased, with the peak fold protein expression observed at 120 minutes, at which point protein levels had increased 2.25-fold from basal levels (Figure 9b).

The effects of the Jak/STAT pathway on the StAR gene

***STAT bind to StAR promoter in vitro* –** As previously stated, upon analysis of the human StAR promoter full and half-binding sites were observed for both STAT1 and STAT3. EMSAs were performed to determine whether STATs bind to the StAR promoter *in vitro*. Binding reactions were prepared containing nuclear extracts from H295R cells, treated with IL-6, and concurrently with IL-6 and AG490 or Piceatannol (Jak and STAT3 inhibitors respectively). Probes used in the binding reactions were custom ordered from Invitrogen to contain the full or double-stranded STAT binding site, within the StAR promoter region (Figure 3). EMSAs showed that a shifted band, retarded from the normal 89 kDa STAT3 band, occurred in those cells treated with IL-6, and was not observed when cells were treated with either of the inhibitors alone (Piceatannol, the STAT inhibitor, showed the greatest decrease). Also, the shifted band was unable to be observed upon simultaneous treatment with IL-6 and AG490 or Piceatannol (Figure 10).

***Binding of STAT to the StAR promoter, increased transcriptional activity of StAR* –** To determine whether binding of STAT to the StAR promoter increased transcriptional activity of StAR *in vivo*, luciferase assays were performed in human adrenal tumor cells (H295R). Constitutively active β-galactosidase plasmid, used as a control, along with pGL2-luc plasmids containing the StAR promoter was transfected in to H295R cells. Cells were then treated with IL-6 alone, Jak or STAT inhibitors alone, and IL-6 plus Jak or STAT inhibitor simultaneously. As shown in Figure 11, when cells were treated with 10pg/ml IL-6, StAR promoter activity and therefore transcription was greatly increased from basal levels, specifically a 5.9-fold increase in RLU

expression was observed with IL-6. Inhibition of either Jak or STAT led to markedly decreased levels of luciferase activity, with a 2.6-fold decrease in activity, from that observed at basal/SF levels seen with treatment of 10μM AG490, and a 4.4-fold decrease was observed upon treatment with 50μM Piceatannol. These results correlated to a decrease in transcriptional activity of the StAR gene upon inhibition of either Jaks or STATs compared to basal levels. When cells were treated with IL-6+AG490, promoter activity levels decreased 4.4-fold from levels observed with IL-6 stimulation. Treatment of cells concurrently with IL-6 and Piceatannol showed markedly decreased levels from IL-6 stimulated activity (36.4-fold). Thus, inhibition of the Jak/STAT pathway at both the Jak and STAT levels decreased StAR promoter activity to basal levels, and IL-6-stimulated promoter activity levels were severely inhibited upon simultaneous treatment with IL-6 and either Jak or STAT inhibitor. Therefore, IL-6 may be acting through the Jak/STAT pathway to mediate its effects upon StAR promoter activity.

***Inhibition of Jak or STAT decreased StAR mRNA levels* –** RT-PCR was performed using mRNA extracted from the human adrenal tumor cell line (H295R) (Figure 12). Cells were treated with 10pg/ml IL-6 alone, 10μM AG490 alone, 50μM Piceatannol alone, and also, AG490 + IL-6 and Piceatannol + IL-6. Fold StAR mRNA levels increased 2.1-fold upon treatment with IL-6 alone. When the Jak inhibitor and the STAT inhibitor were utilized the levels of fold StAR mRNA expression remained unaffected. However upon inhibition of STAT3 by Piceatannol, IL-6-stimulated StAR mRNA levels decreased 1.85-fold, whereas inhibition of Jak by AG490 appeared to not affect IL-6-stimulated StAR mRNA expression. Thus, IL-6 increased mRNA levels

and inhibition of the Jak/STATpathway, specifically STATs, decreased mRNA levels. It should be noted that similar trends were seen upon analysis of promoter activity and protein levels, but since StAR mRNA is highly unstable (6), the effect of Jak/STAT inhibition on mRNA ratios were much less pronounced.

***Inhibition of the Jak/STAT Pathway decreased StAR protein levels* –** Western Blots were performed using human adrenal tumor cells (H295R). Cells were treated with 10pg/ml IL-6 alone, 10μM AG490 alone, 50μM Piceatannol alone, and also, AG490 + IL-6 or Piceatannol + IL-6. StAR protein expression was unaffected when the Jak/STAT pathway was inhibited, by hindering either Jak or STAT function (Figure 13). Also, a 1.5-fold increase in StAR protein level was seen upon treatment with IL-6 alone. When Jak or STAT inhibitors were administered simultaneously with IL-6, a 1.7-fold decrease in protein expression was observed compared that observed upon stimulation with IL-6 alone. Thus, IL-6 increased StAR protein levels and inhibition of the Jak/STAT pathway markedly decreased IL-6-induced StAR protein levels, indicating that IL-6 may possibly be utilizing the Jak/STAT pathway to mediate its effects on StAR protein expression.

DISCUSSION

Effects of IL-6 on StAR Protein, mRNA and promoter activity

Through these experiments we have been better able to elucidate a pathway of steroidogenesis, within human adrenal cells, under conditions mimicking those of chronic stress. As previously stated, IL-6 influences steroidogenesis, specifically increasing cortisol production and release. However the pathway IL-6 utilizes to mediate its effects is not fully understood. IL-6 usually operates through the Jak/STAT pathway, inducing phosphorylation and activation of STATs, which can then translocate into the nucleus to induce transcription of various genes and nuclear factors. However, steroidogenesis is a tightly regulated process, with the rate-limiting step involving the movement of cholesterol into the mitochondria through the action of StAR protein. Thus, these studies focused on the IL-6 pathway and specifically the induction of StAR.

Upon experimentation it can be plainly stated that IL-6 increases StAR promoter activity with subsequent increase in transcription of StAR mRNA and translation of StAR protein. IL-6 was applied to H295R cells at varying levels producing a dose-dependent curve, showing an upward trend in StAR protein, mRNA, and promoter activity upon treatment with as little as 5pg/ml of IL-6, after which a plateau can be observed. Also, cells responded in a time-dependent manner, with a mid-level dose of IL-6 (10pg/ml or 50pg/ml). It was observed that IL-6 increased StAR promoter activity and StAR mRNA expression in a bi-phasic effect, showing to peaks at approximately 30 to 40 minutes and 90 minutes, with a nadir occurring at 60 minutes. StAR protein levels showed a general overall increase upon increasing IL-6 incubation time. As stated above, the StAR promoter region was analyzed and found to contain multiple

STAT binding sites, with only one full STAT binding site. Further experimentations were performed, by inhibiting either Jak or STAT within the Jak/STAT pathway utilized by IL-6, to analyze first whether STAT truly does bind to the StAR promoter, and consequently, whether IL-6 uses this particular pathway to activated the StAR promoter, thus stimulating StAR mRNA and protein levels.

Does STAT bind to the StAR promoter?

Single-stranded DNA probes were custom-made to contain the full STAT1/3 binding site, of the StAR promoter region, to show direct correlation between binding of STATs to StAR and the subsequent increase in steroidogenesis. Upon incubation of H295R cells with IL-6, and subsequent EMSAs, a shifted band was observed, indicating that STAT3 bound to the annealed custom DNA probe. Furthermore, EMSAs demonstrated that inhibition of the Jak/STAT pathway, either at the Jak or STAT level, decreased STAT binding to the StAR promoter sequence since a shifted band was no longer observed. Therefore, it appears that IL-6 increases StAR transcription through activation of the Jak/STAT pathway, inducing the binding of STATs to the StAR promoter region, thereby activating transcription of the StAR gene and thus presumably increasing steroidogenesis.

Effects of Jak/STAT inhibition on StAR Protein, mRNA and promoter activity

Further experiments were performed to show whether the Jak/STAT pathway does indeed influence StAR production, first by inducing expression of the StAR promoter region, then by increasing StAR mRNA, and finally StAR protein levels. It

was observed, through luciferase assays, that 10pg/ml of IL-6 increased StAR promoter activity, and that upon inhibition of the Jak/STAT pathway, relative light units greatly decreased, to basal levels (or lower), and that concurrent incubation with IL-6 and either of the inhibitors minimally increased activity. Thus, it was concluded that IL-6 acts through the Jak/STAT pathway, primarily through the binding of STAT3 to the StAR promoter region, thereby inducing activiation of the StAR promoter. RT-PCRs were then carried out to show whether mRNA levels would correlate to promoter activity shown in the previous luciferase assay results. StAR mRNA levels did increase upon exposure to IL-6 alone, but decreased with either inhibitor and only slightly increased upon the concurrent treatment with IL-6 and either inhibitor. Finally western blots were performed to show a correlation in protein levels to the results previously found. Western blot studies showed a similar trend of increase with IL-6 and a decrease upon inhibition of the Jak/STAT pathway.

As a result of these experimentations, it can be deduced that not only does IL-6 influence steroidogenesis in a time-dependent manner, as well as a dose-dependent manner; it also appears to be bringing about these effects through utilization of the Jak/STAT pathway. Specifically, IL-6 binds to the gp80 subunit of its receptor, thereby dimerizing the two gp130 subunits of the IL-6 receptor. Upon dimerization, phosphorylation of the associated Jak creates docking sites for STATs 1 and 3. When STAT binds to these docking sites, autophosphorylation occurs, thereby allowing STATs to dimerize (creating either homo- or hetero-dimers). Phosphorylated, dimerized STAT then translocates into the nucleus and binds to the STAT binding site located within the StAR promoter region, thereby inducing promoter activity, and thus

inducing transcription of the StAR gene to produce mRNA. Once StAR mRNA is created, translation occurs, and in doing so increases StAR protein levels, thus allowing movement of greater amounts of cholesterol into the inner mitochondria to be enzymatically cleaved, and to consequently increase steroid hormone, namely cortisol, production and release from adrenal cells.

Possible Mechanism, Direct or Indirect Action, of STAT on StAR

While the experimentation above shows that STAT binds to StAR directly and likely influences cortisol production in human adrenal cells, it isn't necessarily explicitly direct in its action. Figure 14 shows the direct and indirect mechanism of cortisol production. IL-6 binds to the gp80/gp130 receptor and phosphorylates the associated Janus Kinases, which in turn phosphorylates the tyrosine heads to create docking sites for STATs. Once STATs dock they are consequently phosphorylated, allowing these active STATs to be released and to homo- or hetero-dimerize, and then translocate into the nucleus. Once inside the nucleus of the adrenal cells, STATs could either be binding directly to the StAR promoter, thereby increasing StAR promoter activity and StAR mRNA and protein expression within the adrenal, which in turn would increase cortisol production; or by the binding of STATs to the promoter of other necessary transcription or nuclear factors important in normal steroidogenesis, such as: Activator Protein-1 (AP-1) components like c-Jun, c-Fos, Jun-B, and Jun-D, or DAX-1 and steroidogeneic factor-1 (SF-1), or possibly even by binding to the crucial enzymes that carry out the necessary cleavage steps to produce cortisol from cholesterol. Therefore, binding of STAT to the promoter of these other steroidogenic-controlling

genes would drive a more indirect mechanism by which IL-6 could be influencing cortisol synthesis. One thing that is clear, STAT3 does indeed bind to the StAR promoter causing an increase in StAR protein expression which then presumbaly brings about an increase in steroid hormone production, specifically that of cortisol. Further experimentation is needed to determine whether IL-6 influences cortisol synthesis directly or whether it also, and more likely, utilizes both direct and indirect means in order to control cortisol, and therefore, steroid hormone production within the adrenal gland during states of chronic stress.

CONCLUSION

StAR protein is crucially important in steroidogenesis; consequently, its expression is tightly regulated by various factors, such as STAT, SF-1 (steroidogenic factor-1) and levels of ACTH, cAMP, and other factors. The StAR gene promoter plays an important role in the regulation of StAR expression. In this study, IL-6 has been shown to increase StAR promoter activity, StAR mRNA, and StAR protein levels within H295R cells, in both time- and dose-dependent manners. Our experiments demonstrate a clear involvement of STATs and the Jak/STAT pathway in relation to the StAR promoter, in the presence and absence of IL-6 stimulation. While, inhibition of the Jak/STAT pathway either by inhibiting Jaks or STAT3 only slight reduces StAR promoter activity, as well as StAR mRNA, and StAR protein from basal levels; IL-6-stimulated increases in StAR promoter activity, StAR mRNA and StAR protein expression were inhibited by antagonists of the Jak/STAT pathway. Therefore, IL-6 increases StAR expression and therefore most likely increasing steroidogenesis by activating the Jak/STAT pathway and subsequently increasing STATs binding to the StAR promoter, which induce transcription of mRNA and thereby increase StAR protein levels. As StAR protein levels increase, more cholesterol can be translocated into the mitochondria, allowing greater steroid hormone production and output.

Further studies need to be performed to determine whether IL-6 induces STAT binding to the promoter of other regulatory molecules, such as DAX-1, SF-1, and AP-1 components to indirectly effect StAR expression, and therefore steroid hormone production. Also, STAT may bind to the promoter of genes necessary for subsequent steps of the pathway, specifically the enzymes necessary for the synthesis of cortisol

from cholesterol, thereby affecting cortisol production within the adrenal gland. By elucidating the pathway used by IL-6 to induce steroidogenesis, a fuller understanding of the role of the adrenal in autoimmune diseases may be discovered. In several autoimmune diseases, including lupus and rheumatoid arthritis, the plasma levels of cortisol are inappropriately low when considering the level of stress of the patient. Through understanding how IL-6 normally regulates cortisol release, we may possibly discover more specific and powerful treatments for these diseases. Greater knowledge of the physiology of disease helps eliminate patients' suffering, as well as increases the understanding of normal cellular functions and the complex pathways they utilize to carry out vital functions.

Figures

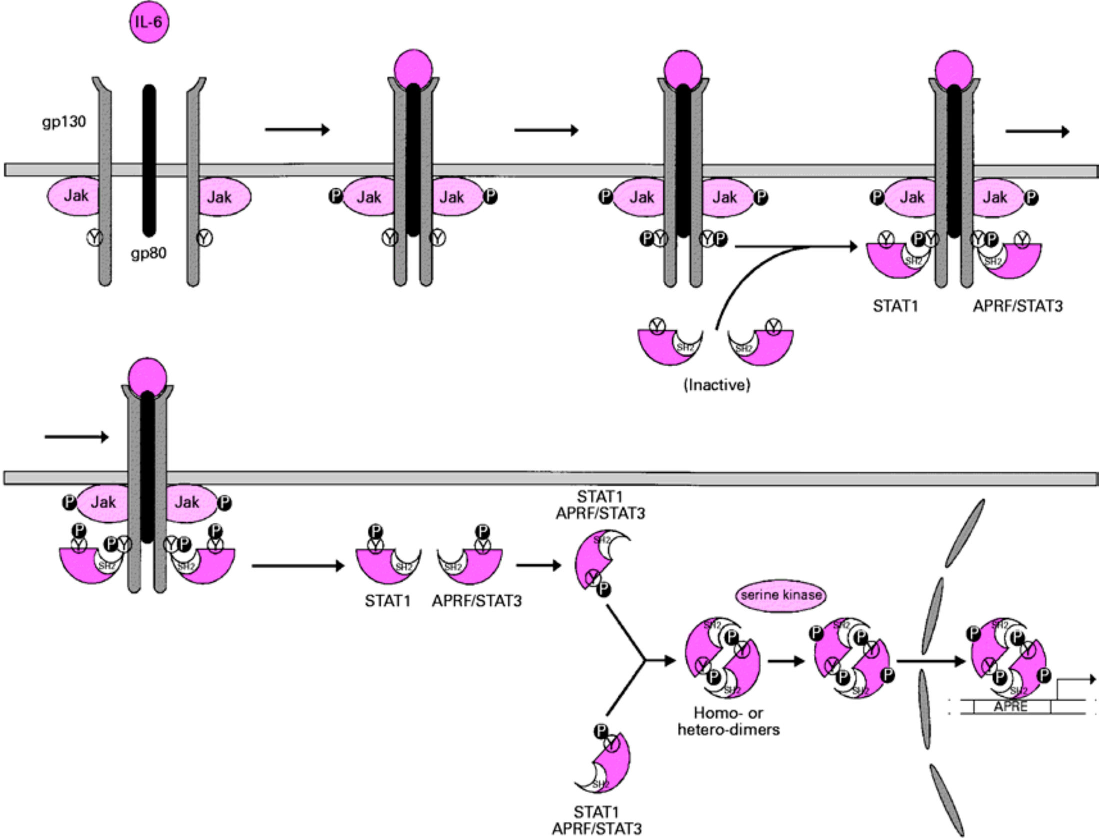


Figure 1: IL-6 induction of gp130/Jak/STAT Pathway (7)
IL-6 mediates its effects during immune responses through the use of the Jak/STAT pathway; therefore IL-6 may be utilizing Jak/STAT to mediate its effects on steroidogenesis within the adrenal gland. In brief, IL-6 binds to gp80 (ligand-binding) subunit, thereby causing dimerization of two gp130 (signal-transducing) subunits. Once they are dimerized, tyrosine sites on Janus Kinases bound to gp130 are phosphorylated, which in turn phosphorylates tyrosine docking sites. These sites once phosphorylated can then dock STAT1 or STAT3. Once docked STATs are phosphorylated and then can dimerize and translocate into the nucleus, where they bind to various gene promoter regions. STAT binding to StAR promoter brings about increased expression of StAR protein, and therefore increased steroidogenesis.

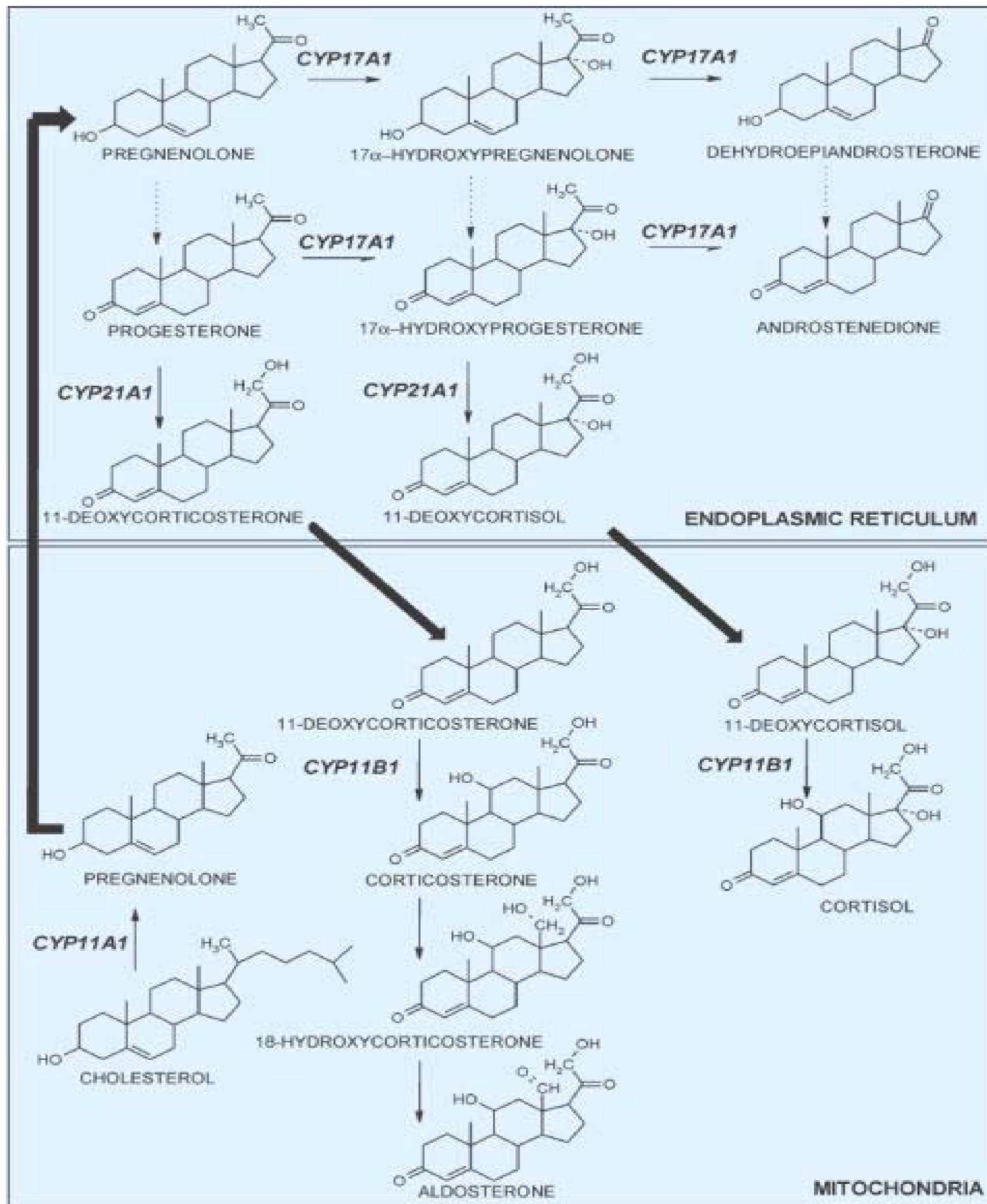


Figure 2: Steroid hormone production pathway (28)
An overview of adrenal steroid hormone production, beginning with cholesterol translocation in to the mitochondria. Cholesterol is cleaved to Pregnenolone, by P450scc (gene designation - CYP11A1), and then subsequent cleavage by various enzymes produce aldosterone, DHEA and cortisol.

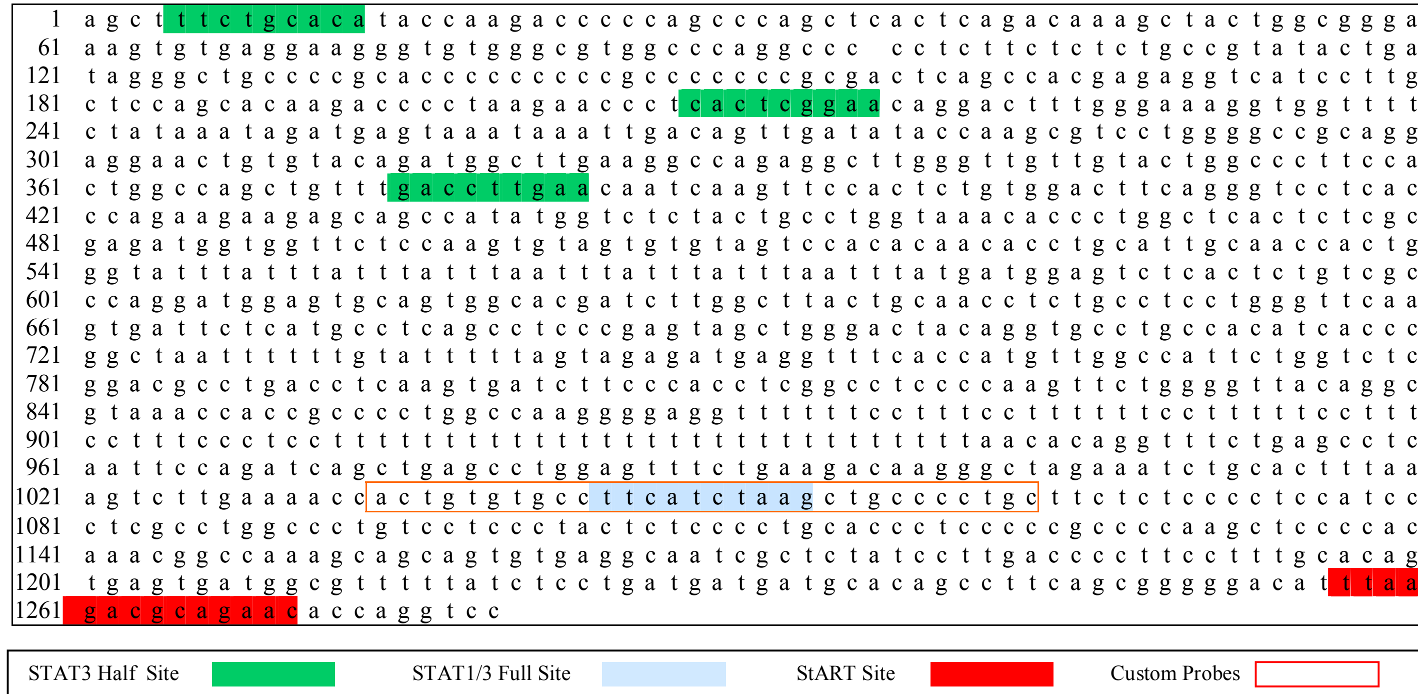


Figure 3: StART Site and STAT Sites of the human StAR gene
Sequence obtained from NCBI of the human StAR gene, STAT sites (both full and half sites) and StART site, were individually obtained and mapped. (Full and half sites refer to the number of DNA strands bound. A full STAT binding site means the STATs bind to both sense and anti-sense strands of the StAR promoter DNA, whereas a half-site binds to one single strand or the other, but not both).

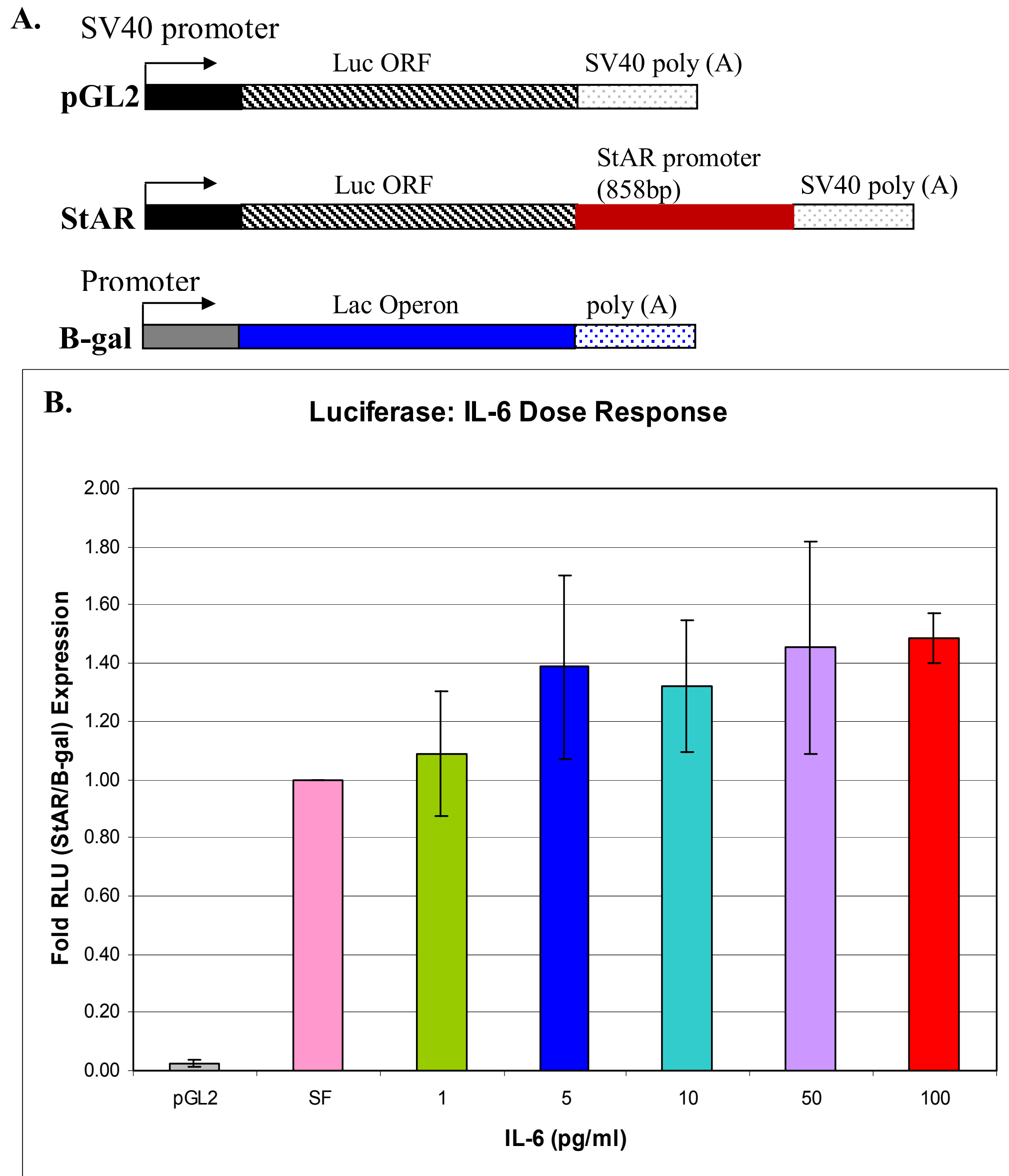
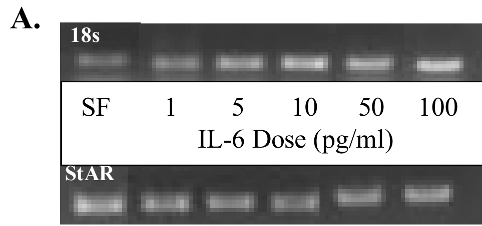


Figure 4: IL-6 activates the StAR promoter in a dose-dependent manner
Briefly, H295R cells were transfected with either pGL2 or co-transfected with StAR and β-gal plasmids. Cells were then treated, in triplicates, with either SF DMEM/F-12 (pGL2 and SF), or increasing amounts of IL-6 (pg/ml) diluted in SF DMEM/F-12. All treatments were incubated for 40 minutes at 37˚C and 5% CO_2. RLUs were calculated by use of luciferase assay reagent or Galacto-Plus β-gal detection systems and measured using a TD-20/20 luminometer. A) Diagram of plasmids transfected into H295R cells. Cells were transfected with either the empty pGL2 plasmid or co-transfected with StAR + β-gal plasmids and then treated. B) Fold Relative Light Unit (RLU) expression (in terms of StAR luciferase activity/ β-gal activity), expressed as fold increase/decrease from Serum-



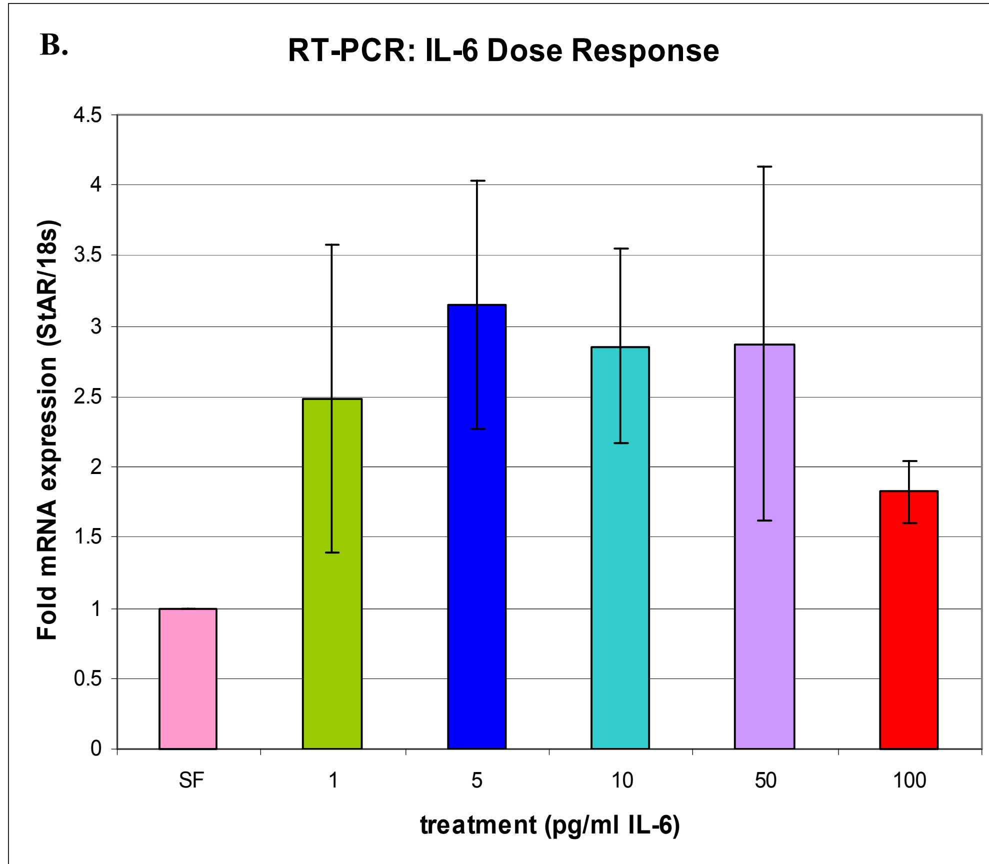
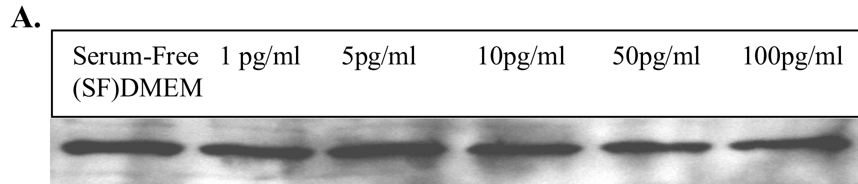


Figure 5: IL-6 increases StAR mRNA levels in a dose-dependent manner
In brief, H295R cells were treated with increasing amounts of IL-6 diluted in serum-free (SF) DMEM/F-12, for 4 hours at 37˚C and 5% CO_2. mRNA was extracted and RTPCR performed to determine the levels of mRNA expression. Custom Human StAR primer sequences were used along with standard Classic 18s primers to amplify the DNA. SF denotes serum free DMEM/F-12 media, which was used for SF/basal levels. Extractions, and therefore RTPCR experiments, were run in triplicates. Fold mRNA expression was calculated as a ratio of StAR to 18s levels and measured through use of the SpotDenso tool on the FluorChem 8900 Imaging System, and adjusted for background. A) RT-PCR results after gel-electrophoresis and detection by UV. B) Fold mRNA expression (ratio of StAR/18s), expressed as fold increase/decrease from Serum-Free (SF)/basal levels. Error is ±SEM of three replicates.

A.



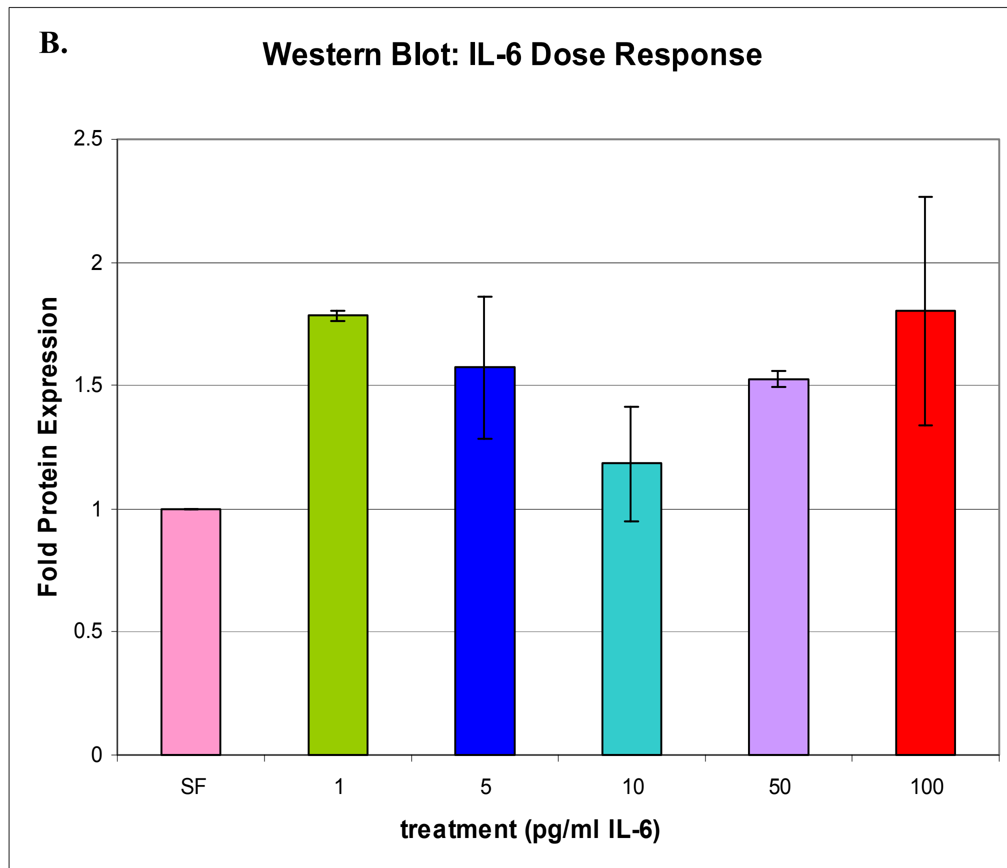
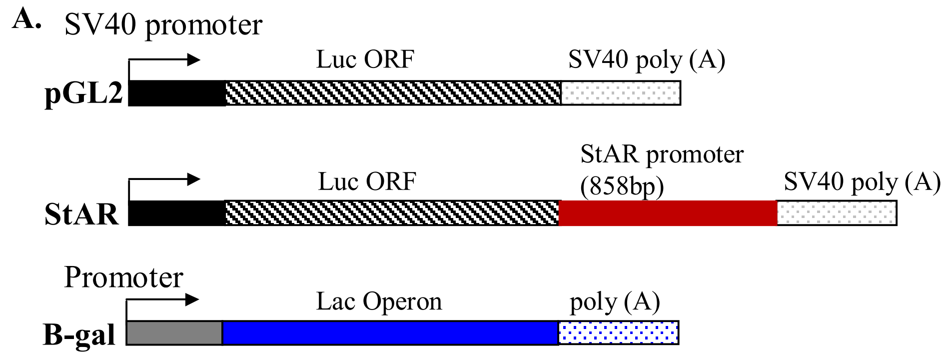


Figure 6: IL-6 mediates a dose-dependent increase in StAR protein levels in H295R cells H295R cells were treated, with increasing amounts of IL-6 diluted in serum-free (SF) DMEM/F-12, for 60 minutes at 37˚C and 5% CO_2. Protein was then extracted and western blots performed to determine the levels of protein expression. Primary StAR Antibodies (Santa Cruz) were used to detect StAR protein levels. Extractions and subsequent western blot experiments were run in triplicates. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the SpotDenso tool on the FluorChem 8900 Imaging System and each band was adjusted for band-specific background. A) Western blot results after electrophoresis, transfer, antibody blotting and subsequent detection by luminol/stable peroxidase. B) Fold protein expression were calculated as fold increase/decrease from SF/basal levels. Error is ±SEM of three replicates.



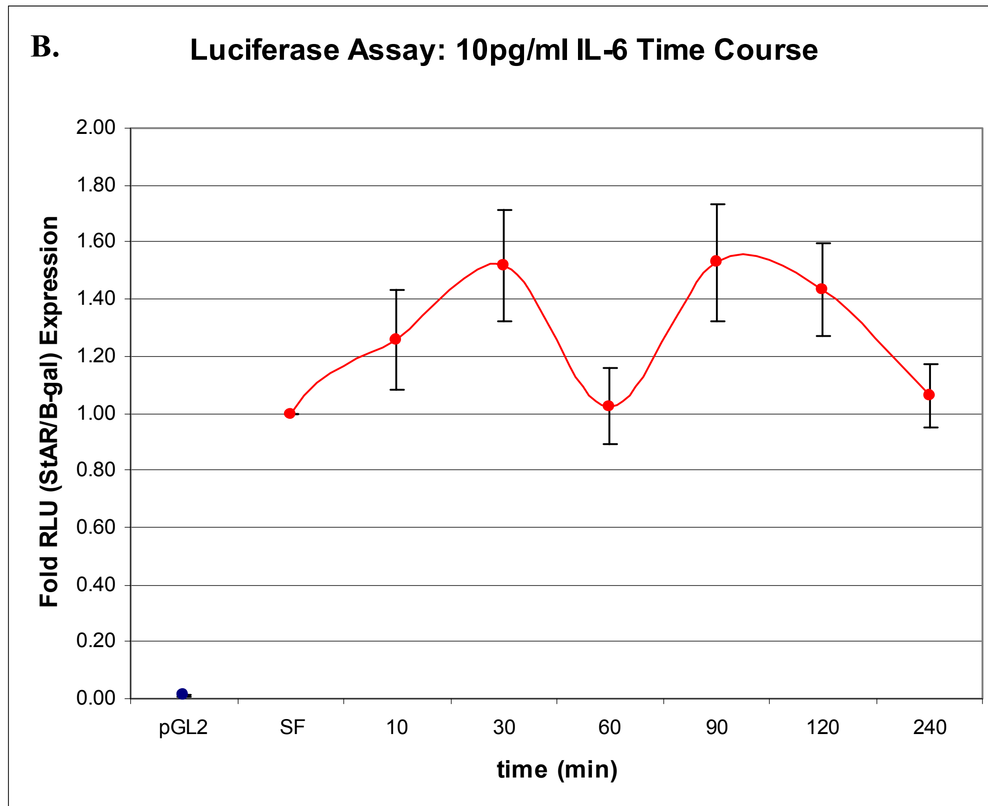
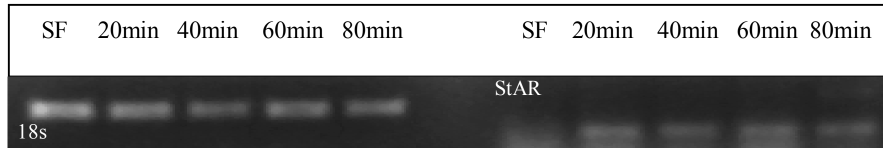


Figure 7: Activation of the StAR promoter by IL-6 is time-dependent
Briefly, in 12-well plates, H295R cells were transfected with either pGL2 or co-transfected with StAR and β-gal plasmids. Cells were then treated with serum-free (SF) DMEM/F-12 (pGL2 and SF) for 240 minutes, or 10pg/ml of IL-6 diluted in SF DMEM/F-12 for various amounts of time at 37˚C and 5% CO_2. RLUs were calculated by use of luciferase assay reagent and Galacto-Plus β-gal detection systems and measured using a TD-20/20 luminometer. A) Diagram of plasmids transfected into H295R cells. Cells were transfected with either the empty pGL2 plasmid or co-transfected with StAR + β-gal plasmids and then treated. B) Fold Relative Light Unit (RLU) expression (in terms of StAR luciferase activity/ β-gal activity), expressed as fold increase/decrease from SF levels with ±SEM of four replicates.



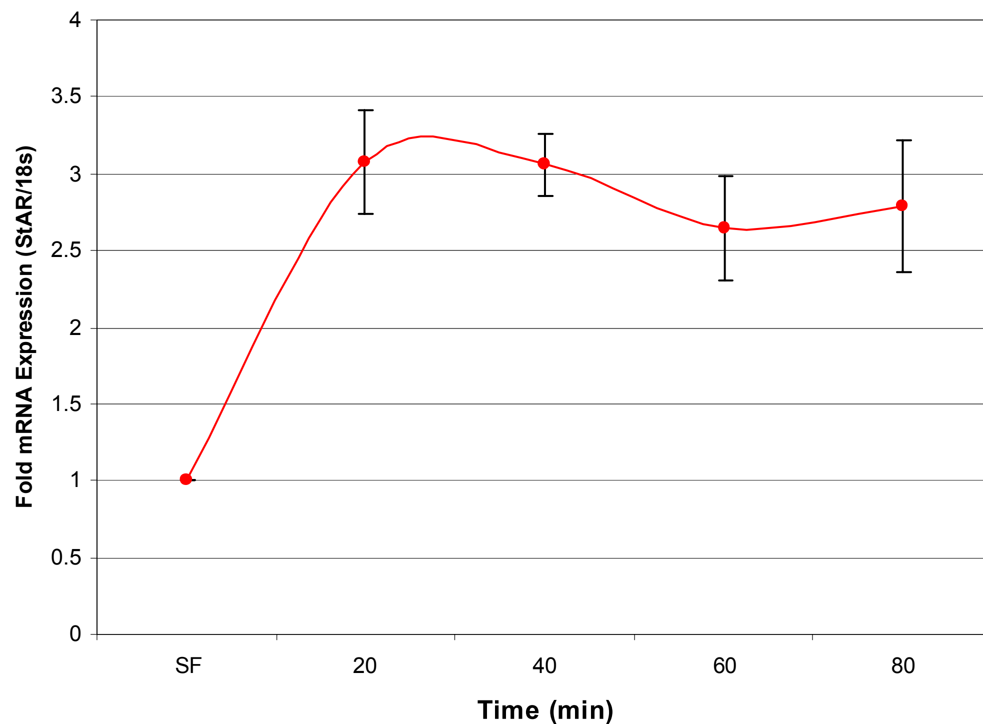


Figure 8: IL-6 induces StAR mRNA production in a time-dependent manner
H295R cells were treated, with 10pg/ml IL-6 diluted in serum-free (SF) DMEM/F-12 media, for increasing amounts of time at 37˚C and 5% CO_2. mRNA was extracted an d RTPCR performed to determine mRNA levels. Custom Human StAR primer sequences were used along with standard Classic 18s primers to amplify the DNA. Treatments, extractions and RTPCR were run in duplicates. Fold mRNA expression was calculated as a ratio of StAR to 18s levels and measured through use of the SpotDenso tool on the FluorChem 8900 Imaging System and adjusted for background. A) RT-PCR results after gel-electrophoresis and detection by UV. B) Fold mRNA expression (ratio of StAR/18s), expressed as fold increase/decrease from SF/basal levels. Error is ±SEM of two replicates.

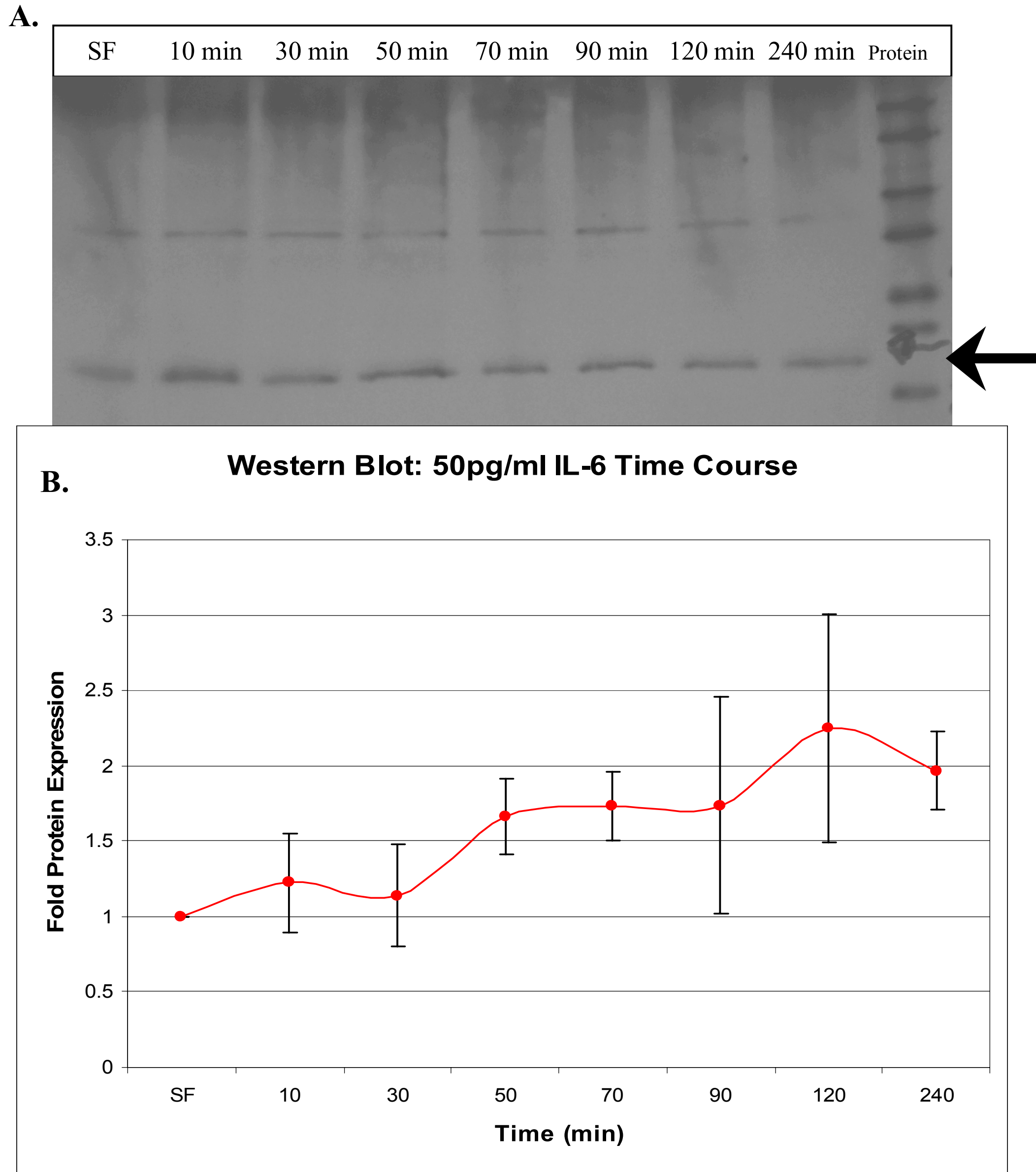


Figure 9: IL-6 induces increased StAR protein levels in a time-dependent manner
H295R cells were treated, with 50pg/ml of IL-6 diluted in serum-free (SF) DMEM/F-12, for increasing amounts of time at 37˚C and 5% CO_2 (treatment with SF DMEM with no IL-6 were carried out for 240 minutes). Protein was then extracted and western blots performed to determine the levels of protein expression. Primary StAR Antibodies (Santa Cruz) were used to detect StAR protein levels. Extractions and subsequent western blot experiments were run in triplicates. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the SpotDenso tool on the FluorChem 8900 Imaging System and each band was adjusted for band-specific background. A) Western blot results after electrophoresis, transfer, antibody blotting and subsequent detection by luminol/stable peroxidase. Black arrow denotes the 30kDa StAR band analyzed. B) Fold protein expression were calculated as fold increase/decrease from SF/basal levels. Error is ±SEM of either three replicates (SF-70 minutes) or two replicates (90-240 minutes).

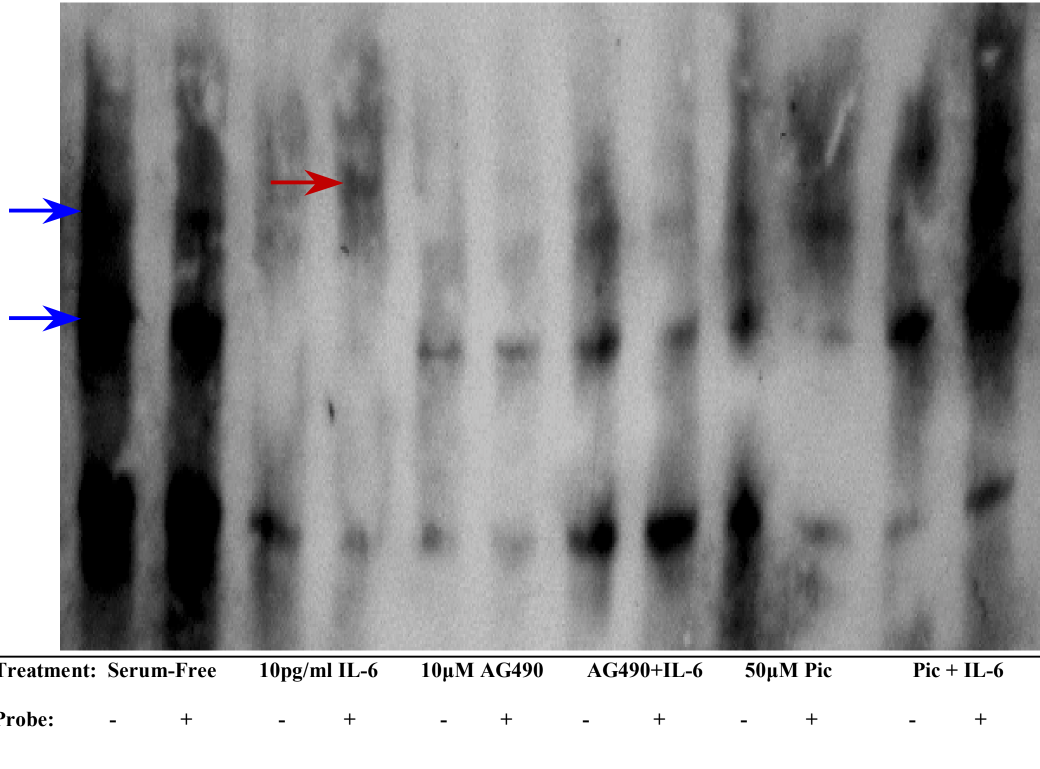
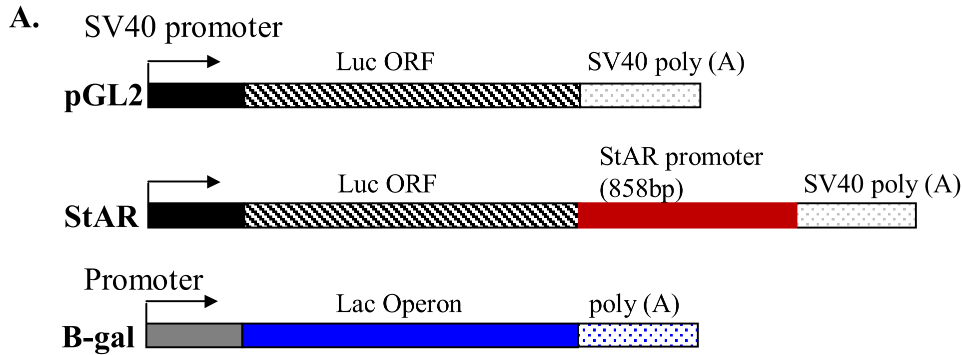


Figure 10: STAT3 binds to StAR gene to promote transcription
H295R cells were treated with serum-free DMEM/F-12 (SF), 10pg/ml of IL-6, 10µM AG490 (Jak Inhibitor), 10µM AG490+10pg/ml IL-6, 50µM Piceatannol (STAT3 Inhibitor), or 50µM Piceatannol+10pg/ml IL-6 at 37˚C and 5% CO_2 for 40 minutes. Nuclear proteins were then extracted and Electromobility shift assays (EMSA) performed to determine whether STAT binds to the StAR promoter region. Custom StAR promoter DNA probes containing the full STAT binding site were first annealed and then bound to the nuclear extracts at room temperature for 20 minutes. Binding reactions were then electrophoresed and detected by primary STAT3 antibodies and luminol/stable peroxidase detection system (Santa Cruz). Each reaction was run in duplicate in order to properly compare, with one reaction (most right of each couplet) containing only nuclear extract, and the other (left lane of each couplet) containing nuclear extract+ds-DNA probe.



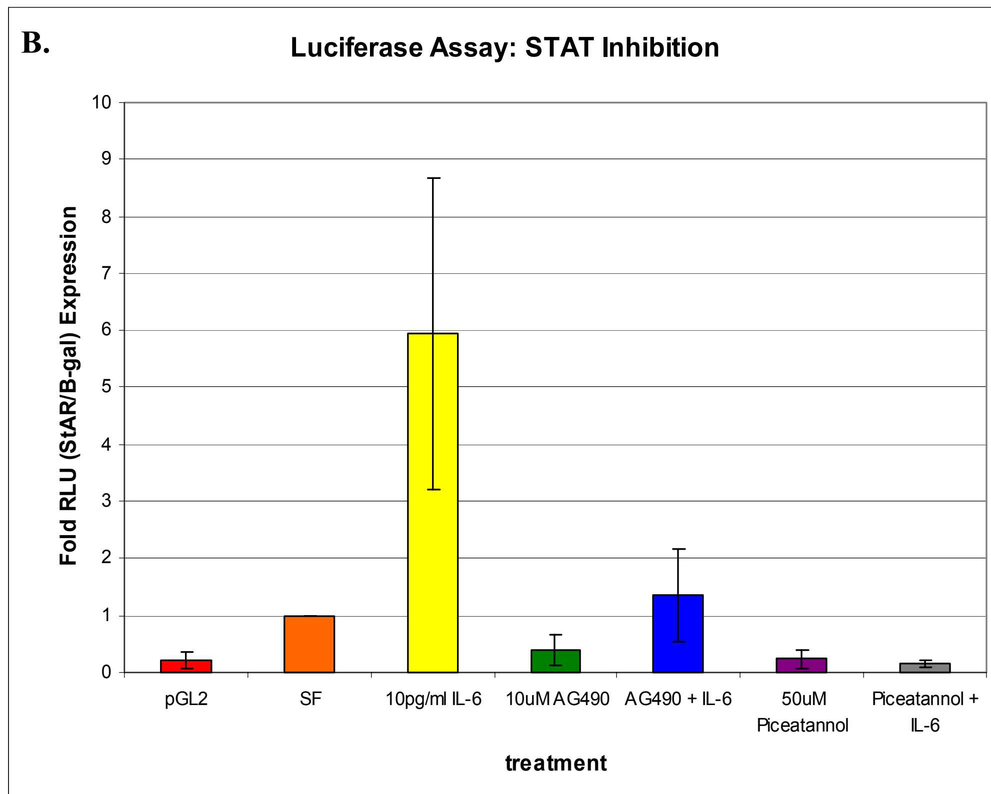
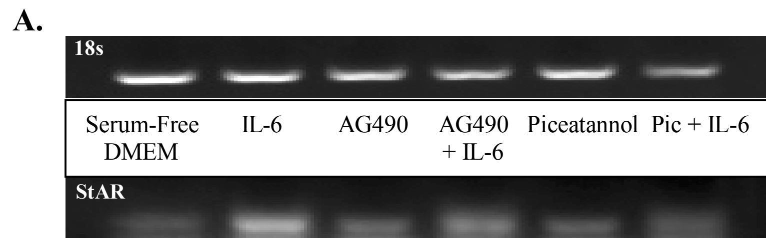


Figure 11: IL-6 utilizes the Jak/STAT pathway to activate the StAR promoter
In brief, H295R cells were transfected with either pGL2 or co-transfected with StAR and β-gal plasmids. Cells were then treated with serum-free (SF) DMEM/F-12 (pGL2 and SF), or 10pg/ml IL-6, 10μM AG490 (Jak Inhibitor), 10μM AG490+10pg/ml IL-6, 50μM Piceatannol (STAT3 inhibitor), or 50μM Piceatannol+10pg/ml IL-6, all of which were diluted in SF DMEM/F-12. Cells were incubated with treatments for 40 minutes, at 37˚C and 5% CO_2. RLUs were calculated by use of luciferase assay reagent and Galacto-Plus β-gal detection systems and measured using a TD-20/20 luminometer. A) Diagram of plasmids transfected into H295R cells. Cells were transfected with either the empty pGL2 plasmid or co-transfected with StAR + β-gal plasmids and then treated. B) Fold Relative Light Unit (RLU) expression (in terms of StAR luciferase activity/ β-gal activity), expressed as fold increase/decrease from Serum-Free (SF) levels. Errors are ±SEM of three replicates.

A.



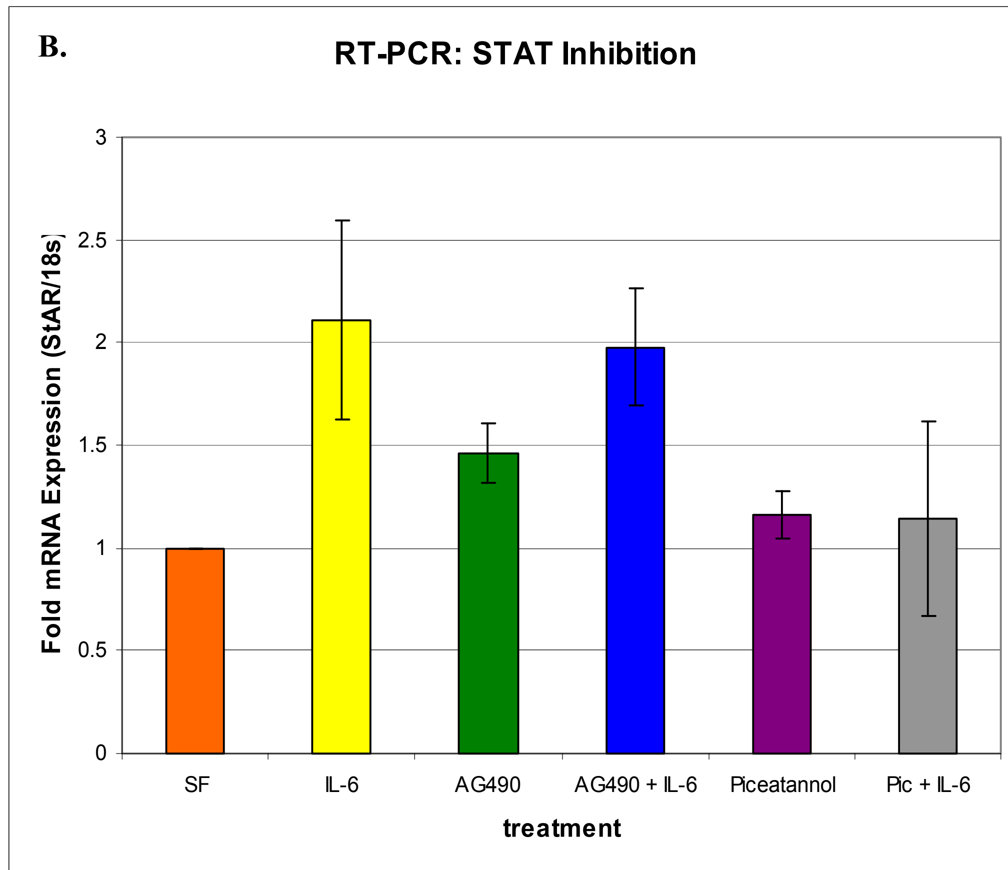
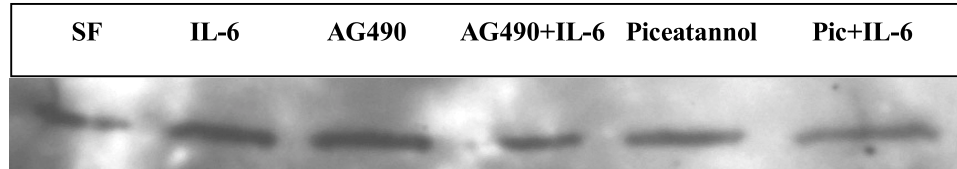


Figure 12: IL-6 increases StAR mRNA levels through the Jak/STAT Pathway
H295R cells were treated with serum-free DMEM/F-12 (SF), 10pg/ml of IL-6, 10μM AG490 (a specific Jak Inhibitor), 10μM AG490+10pg/ml IL-6, 50μM Piceatannol (specific STAT 3 inhibitor), or 50μM Piceatannol+IL-6 for 40 minutes at 37˚C and 5% CO_2. mRNA was extracted an d RTPCR performed to determine mRNA levels. Custom Human StAR primer sequences were used along with standard Classic 18s primers to amplify the DNA. Treatments, extractions, and RTPCR were run in duplicates. Fold mRNA expression was calculated as a ratio of StAR to 18s levels, measured through use of the SpotDenso tool on the FluorChem 8900 Imaging System, and adjusted for background. A) RT-PCR results after gel-electrophoresis and detection by UV. B) Fold mRNA expression (ratio of StAR/18s), expressed as fold increase/decrease from SF/basal levels. Error is ±SEM of three replicates.

A.



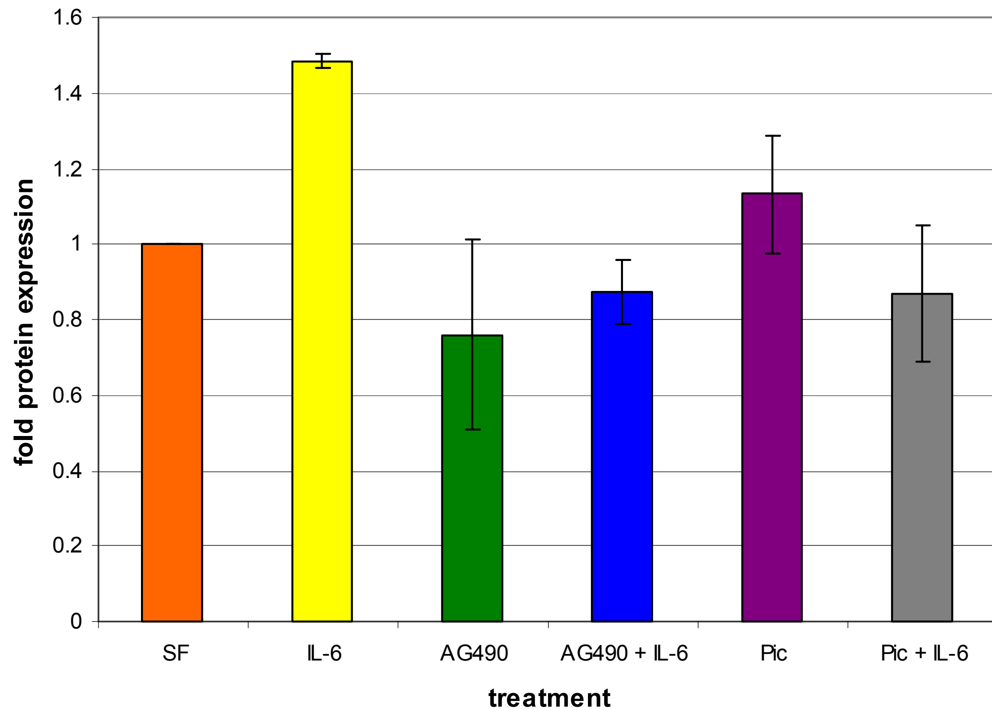


Figure 13: IL-6 induces StAR protein expression through use of the Jak/STAT pathway H295R cells were treated with serum-free DMEM/F-12 (SF), 10pg/ml of IL-6, 10µM AG490 (a specific Jak Inhibitor), 10µM AG490+10pg/ml IL-6, 50µM Piceatannol (specific STAT 3 inhibitor), or 50µM Piceatannol+IL-6 for 40 minutes at 37˚C and 5% CO_2. Protein was then extracted and western blots performed to determine the levels of protein expression. Primary StAR Antibodies (Santa Cruz) were used to detect StAR protein levels. Extractions and subsequent western blot experiments were run in triplicates. Fold protein expression was detected using luminol/stable peroxidase detection system (Santa Cruz). Levels were measured through use of the SpotDenso tool on the FluorChem 8900 Imaging System and each band was adjusted for band-specific background. A) Western blot results after electrophoresis, transfer, antibody blotting and subsequent detection by luminol/stable peroxidase. B) Fold protein expression were calculated as fold increase/decrease from SF/basal levels. Error is ±SEM two replicates.

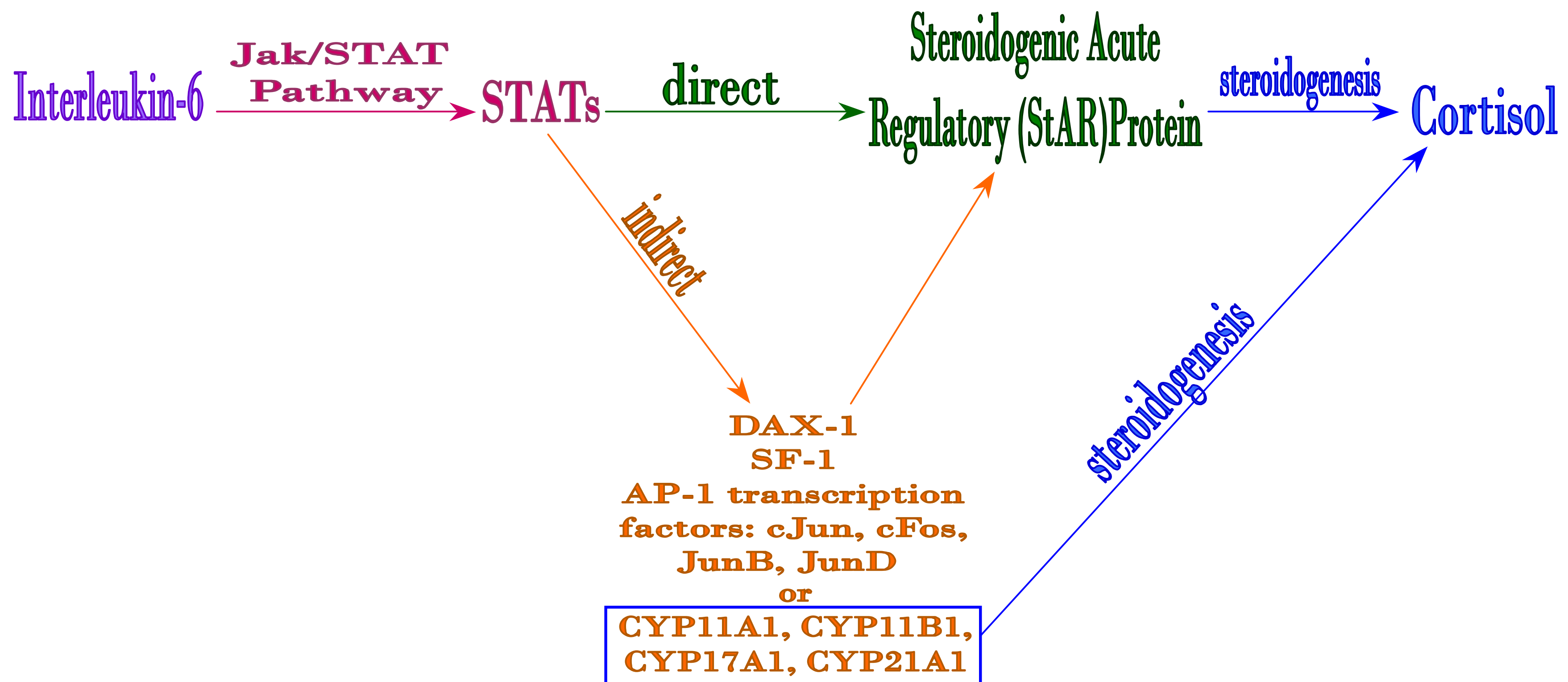


Figure 14: Possible Mechanisms of Action Utilized by IL-6 to Stimulate Cortisol Production
Interleukin-6 (IL-6) utilizes the Jak/STAT pathway to mediate its effects on steroidogenesis. Upon stimulation of the Jak/STAT pathway, active and dimerized STATs translocate into the nucleus and either could bind directly to StAR (which has been shown by EMSA studies), but could possibly also carry out its steroidogenic influences by binding to various nuclear factors, or transcription factors known to control various aspects of steroid hormone production. DAX-1 has been shown in our lab (data not included) to decrease StAR expression, while SF-1 increases StAR, AP-1 also acts to increase StAR expression. STATs could also bind to various cytochrome P450 genes important in production of the enzymes necessary for cortisol synthesis in the mitochondria, thus acting to regulate steroidogenesis independent of StAR.

Literature Cited

1. Arakane, F., Sugawara, T., Nishino, H., Liu, Z., et al. (1996). Steroidogenic acute regulatory protein (StAR) retains activity in the absence of its mitochondrial import sequence: Implications for the mechanims of StAR action. *Biochemistry* **93**, 13731-13736.

2. Bornstein, S.R., Rutkowski, H., Vrezas, I. (2002). Cytokines and steroidogenesis. *Molecular and Cellular Endocrinology* **215**, 135-141.

3. Buettner, R., Mora, L.B., Jove, R. (2002) Activated STAT signaling in Human Tumors Provides Novel Molecular Targets for Therapeutic Intervention. *Clincal Cancer Research* **8**, 945-954.

4. Candotti, F., Notarangelo, L., Visconti, R., O'Shea, J. (2002) Molecular aspects of primary immunodeficiencies: lessons from cytokine and other signaling pathways. *The Journal of Clinical Investigation* **109**, 1261-1269.

5. Decker, T., Stockinger, S., Karaghiosoff, M., Muller, M., and Kovarik, P. (2002). IFNs and STATs in innate immunity to microorganisms. *The Journal of Clincial Investigation* **109**, 1271-1277.

6. Haichuan, Duan and Jefcoate, Colin R. (2007). The predominant cAMP-stimulated 3-5kb StAR mRNA contains specific sequence elements in the extended 3'UTR that confer high basal instability. *Journal of Molecular Endocrinology* **38**, 159-179.

7. Heinrich, P., Behrmann, I., Muller-Newen, G., Schaper, F., Graeve, L. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. Journal* **334**, 297-314.

8. Judd, Allan M., et al. (1998). Possible Function of IL-6 and TNF as Intraadrenal Factors in the Regulation of Adrenal Steroid Secretion. *Annals of the New York Academy of Sciences* **917**, 628-637.

9. Kanazaki, Masanori and Morris, Patricia. (2004). Growth Hormone Regulates Steroidogenic Acute Regulator Protein Expression and Steroidogenesis in Leydig Cell Progenitors. *Endocrinology* **140**, 1681-1686.

10. Kelly, SN, McKenna, JT, Young, LS. (2004). Modulation of steroidogenic enzymes by orphan nuclear transciptional regulation may control diverse production of cortisol and androgens in the human adrenal. *Journal of Endocrinology* **181**, 355-365.

11. Levy, David E., Lee, Chein-kuo. (2002). What does Stat 3 do? *The Journal of Clinical Investigation* **109**, 1143-1148.

12. Luo, Hong and Dearolf, Charles R. (2001). The Jak/STAT pathway and Drosophila devlopment. *BioEssays* **23**, 1138-1147.

13. Manna, PR, Dyson, MT, Eubank, DW, et al. (2002). Regulation of Steroidogenesis and the Steroidogenic Acute Regulatory Protein by a member of the cAMP Response-Element Binding Protein Family. Molecular Endocrinology **16**, 184-199.

14. Manna, Pulak R., Eubank, Darrell W., and Stocco, Douglas M. (2004). Assessment of the Role of Activator Protein-1 on Transcription of the Mouse Steroidogenic Acute Regulatory Protein Gene. *Moecular Endocrinology* **18**, 558-573.

15. Osman, H., Murigande, C., et al. (2002). Repression of DAX-1 and Induction of SF-1 Expression. *The Journal of Biological Chemistry* **227**, 41259-41267.

16. Porter, JP. (1999). Chronic intracerebroventricular infusion of angiotensin II increases brain AT1 receptor expression in young rats. *Developmental Brain Research* **112**, 239-295.

17. Ruiz-Cortes, Z.T., et al. (2003) Biphasic Effects of Leptin in Porcine Granulosa Cells. *Biology of Reproduction* **68**, 789-796.

18. Schindler, Christian W. (2002). Jak-STAT signaling in himan diesease. *The Journal of Clincal Investigation* **109**, 1133-1137.

19. Silverman, Marni N., Miller, Andrew H., Biron, Christine A., Pearce, Brad D. (2004). Characterization of an Interleukin-6 and Adrenocorticotropin-Dependent, Immune-to-Adrenal Pathway during Viral Infection. *Endocrinology* **145 (8)**, 3580-3589.

20. Stocco DM. (2001). StAR protein and the regulation of steroid hormone biosynthesis. *Annual Reviews of Physiology* **63**, 193-213.

21. Stocco, DM. (2000). The role of the StAR protein in steroidogenesis: challenges for the future. *Journal of Endocrinology* **164**, 247-253.

22. Stocco, Douglas M. (1998). Recent advances in the role of StAR. *Journals of Reproduction and Fertility* **3**, 82-85.

23. Stocco, Douglas M. (2000). Tracking the Role of a StAR in the Sky of the New Millennium *Molecular Endocrinology.* **15(8)**, 1245-1254.

24. Straub, Rainer H., et al. (2002). Dehydroepiandrosterone in relation to other adrenal hormones during an acute inflammatory stressful disease state compared with chronic inflammatory disease: role of interleukin-6 and tumour necrosis factor. *European Journal of Endocrinology* **146**, 365-374.

25. Sugawara, T., Hoshi, M., Sakuragi, N. (2004). Immunohistochemical Study of Steroidogenic Acute Regulator (StAR) Protein in Ovarian Tumors. *The Internet Journal of Gynecology and Obstetrics* **3**.

26. Taketa, Kathryn M. (2004). The Role of Interleukin-6 Regulation of Steroidogenic Acute Regulatory Protein in Cortisol and DHEA Secretion from Bovine Adrenal Tissue and Human Adrenal Tumor H295R Cells. Honors Thesis, Brigham Young University.

27. Tsujishita, Yosuke and Hurley, James H. (2000). Structure and lipid transport mechanism of a StAR-related domain. *Nature Structural Biology.* **7(5)**, 408-14.

28. Zuber, R., Anzenbacherova, Eva, Anzenbacher, P. (2002). Cyctochromes P450 and experimental models of drug metabolism. *Journal of Cellular and Molecular Medicine*. **6(2)**, 189-19.

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