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REGULATION OF ADRENAL STEROIDOGENESIS BY INTERLEUKIN-6

By

Stephen McIlmoil

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

Stephen A. McIlmoil

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

Date

Allan M. Judd, Chair

Date

William W. Winder

Date

Eric Wilson

BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the thesis of Stephen McIlmoil 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.

Date

Allan M. Judd,
Chair, Graduate Committee

Accepted for the Department

Dixon J. Woodbury
Graduate Coordinator

Accepted for the College

Rodney J. Brown
Dean, College of Life Sciences

ABSTRACT

REGULATION OF ADRENAL STEROIDOGENESIS BY INTERLEUKIN-6

Stephen A. McIlmoil

Physiology and Developmental Biology

Master of Science

Cortisol and dehydroepiandrosterone (DHEA) are steroids produced by the zona fasciculata (ZF) and reticularis (ZR), respectively, of the adrenal cortex. Both steroids are upregulated in response to adrenocorticotrophic hormone (ACTH). Cortisol is a glucocorticoid that is important in the regulation of inflammation and metabolism. DHEA is an adrenal androgen important in fetal growth and puberty but tends to decrease gradually after puberty in both men and women. DHEA has various effects on metabolism and immune function including inhibiting the effects of cortisol on some tissues.

During the acute phase of stress, cortisol and DHEA rise due to an increase in ACTH released from the anterior pituitary. In contrast, during chronic stress, cortisol remains elevated but DHEA and ACTH levels decrease. Likewise, stress causes serum levels of IL-6 to increase. IL-6 increases cortisol release from the human and bovine adrenal cortex. IL-6 also decreases DHEA release from zona reticularis of the bovine adrenal gland. In humans the effect of IL-6 on DHEA production is still uncertain.

To determine a possible mechanism of IL-6 on the zona fasciculata and reticularis, human H294R cells and bovine adrenal tissue were incubated in serum free

medium containing IL-6, at various concentrations and incubation intervals. At the end of the incubation interval, mRNA or protein was extracted from the cells or tissue. Standard PCR, real time PCR, and western blot assays were used to determine the effects of IL-6 on the enzymes involved in cortisol and DHEA synthesis, steroidogenic factor-1 (SF-1), steroidogenic acute regulatory protein (StAR), and dosage sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (DAX-1).

In human H295R cells and bovine zona fasciculata cells IL-6 caused an increase in SF-1, StAR, P450_{scc}, 17 α hydroxylase, 3 β -hydroxysteroid dehydrogenase type 2 (3 β HSD2), 21 hydroxylase, and 11 β hydroxylase mRNA and protein. IL-6 caused DAX-1 mRNA and protein to decrease. These effects were manifest in a time dependent manner. Dose response treatments incubated for 60 min increased SF-1, StAR, P450_{scc}, 17 α hydroxylase, 3 β HSD2, 21 hydroxylase, and 11 β hydroxylase but there was not significant change between the different treatments of IL-6. The bovine zona reticularis stimulated with IL-6 showed a decrease in SF-1, StAR, P450_{scc}, 17 α hydroxylase, and 3 β HSD2 with an increase in DAX-1 mRNA and protein. This response was manifest in a time dependent manner for both mRNA and protein, and the effect was dose-dependent for mRNA but not protein levels within the 60 min time period. These data provide a mechanism by which increased stress, physical or emotional, which increases IL-6 serum level, could increase cortisol and decrease DHEA. This would account for decreased immune function, increased blood pressure, and changes in metabolism.

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Introduction

Glucocorticoids are steroid hormones that regulate serum glucose levels. Cortisol is the main glucocorticoid produced by the body. Cortisol is released from the zona fasciculata of the adrenal cortex during stress and is derived from cholesterol. Cortisol affects many different tissues and processes of the body. Some effects of cortisol include 1) decreasing the number of circulating lymphocytes 2) inhibiting T-cell activation and maturation 3) increasing gluconeogenesis, protein breakdown, and increasing amino acids in the blood and fatty acid liberation 4) increasing blood pressure by causing vasoconstriction due to an increase in alpha adrenergic receptors 5) increasing renal blood flow and glomerular filtration rate 6) increasing Na^+ reabsorption by the collecting duct 7) decreasing water absorption 8) decreasing bone formation rate and increasing demineralization (25).

Cortisol is produced by conversion of cholesterol to the active steroid. Cholesterol is made available for conversion to steroids by adrenal via endocytosis of a low density lipoprotein (LDL) (26), synthesis of cholesterol by the adrenal (27), selective cellular uptake (27), stored cholesterol esters inside the adrenal (26), and scavenger receptors (28). Intercellular cholesterol that will be used in steroidogenesis is transported from the cytosol to the intermembrane space of the mitochondria by the steroidogenic acute regulatory protein (StAR) (24). In the mitochondria the side chain of cholesterol is cleaved, by cytochrome P450 side chain cleavage enzyme (P450_{scc} or desmolase), producing pregnenolone. Pregnenolone then leaves the mitochondria, entering cytoplasm, where it is converted to progesterone in the by 3β hydroxysteroid dehydrogenase type 2 (3β HSD2). Progesterone, in the endoplasmic reticulum, is enzymatically converted by 17α hydroxylase (P450_{c17}) to 17OH -progesterone. Further modifications then happen in the endoplasmic reticulum by adding a hydroxyl group to the 21 carbon by 21 hydroxylase (P450_{c21}). The final step of cortisol production occurs in the mitochondria, where the 11 carbon is hydroxylated by 11β hydroxylase (P450_{c11 β}) (18) (Diagram 1).

The traditional pathway of stimulation of cortisol production is through corticotrophin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH). CRH is released from the hypothalamus which enters the hypophyseal portal system via capillary beds in the median eminence, travels through the blood down the long portal veins, and enters the anterior pituitary where it acts on corticotrophs causing the release of ACTH. ACTH then enters the blood stream and flows to the adrenal where it binds to a seven transmembrane receptor. This receptor, when activated by ACTH, causes the activation of a G-protein, G_s . G_s then activates adenylyl cyclase and cAMP is produced. cAMP binds to regulatory subunits of PKA which activates the kinase (30). PKA phosphorylates proteins such as hormone sensitive lipase (HSL) (29) and cAMP response element binding protein (CREB). Phosphorylated CREB translocates to the nucleus and causes transcription (31). Through these actions, cortisol is produced.

Acute stress causes a rise in ACTH and therefore, cortisol. During chronic stress, the concentration of ACTH does not correspond to the concentration of cortisol (13). Therefore, other hormones or cytokines must be causing the elevated cortisol levels. One cytokine that has been shown to cause cortisol secretion is interleukin-6 (IL-6) (10). IL-6 is produced by many tissues in the body including activated T-cells, monocytes, fibroblasts, endothelial cells, macrophages and skeletal muscle. It is also produced by the zona glomerulosa of the rat adrenal and all zones of the human and bovine adrenal cortex (10). IL-6 produced by the adrenal could have a paracrine effect on the adrenal affecting the zona fasciculata. The adrenal cortex has been shown to increase IL-6 production when stimulated by IL-1 α , IL-1 β , TNF α , ACTH, and angiotensin II (10).

Dehydroepiandrosterone (DHEA) is an androgen produced and released from the zona reticularis of the adrenal cortex. ACTH has been shown to increase the production of DHEA in healthy human subjects (19). IL-6 has been shown to cause a decrease in the basal level and ACTH stimulation of DHEA production in bovine adrenal cells (20). DHEA is thought of as a prohormone. DHEA is secreted in large amounts and is converted to sex steroids locally in the tissue. In this manner the tissue can regulate the amount of steroid and when it will be stimulated by the steroid

in a precise manner. DHEA indirectly effects bone formation, adipose tissue, muscle, insulin and glucose metabolism, skin, and libido (23).

The IL-6 regulation of steroidogenesis in the adrenal cortex is thought to be mediated by the janus kinase (JAK) system (unpublished observation). In other tissues, IL-6 effects are mediated through the JAK-STAT (signal transducers and activator of transcription protein) pathway. Some of these effects include increased body temperature and regulation of inflammation. The JAK associated with the IL-6 receptor is a serine-threonine kinase. When IL-6 binds to the receptor, the receptor dimerizes and JAK phosphorylates the cytoplasmic tails of the IL-6 gp130 subunit. JAK subunits are auto-phosphorylated when IL-6 binds to the receptor. When the JAK is phosphorylated, STATs bind near the phosphate group on the gp130 subunits and are phosphorylated by JAK. Phosphorylated STATs then dimerize. Dimerized STATs move to the nucleus and cause the transcription of genes. The dimerized STATs can be homo- or hetero-dimers (14, 21).

CREB and the STATs are nuclear factors. Nuclear factors are proteins which regulate the rate of transcription of genes. STATs are involved in steroidogenesis in bovine luteal cells (15). CREB is involved in the regulation of genes but the role of CREB in steroidogenesis is uncertain. It has been suggested that the acute phase 1 proteins (AP-1), specifically cFos and cJun, play a larger role and cooperate with steroidogenic factor-1 (SF-1) to cause transcription of cytochrome P450 side chain cleavage enzyme (16). SF-1 has also been shown to be important for steroidogenesis in many tissues. While SF-1 up-regulates transcription of steroidogenic components, DAX-1 (Dosage sensitive sex reversal – adrenal hypoplasia congenita gene on the X chromosome, gene 1) has an opposing effect (3). DAX-1 is a nuclear factor which suppresses transcription. In mouse adrenal cells Dax-1 inhibits StAR and decreases P450_{scc} and 3 β HSD2 promoter activity (17).

IL-6 increases cortisol production in bovine ZF cells and human H295R cells, but decreases DHEA production in bovine ZR cells (20). This paradoxical effect of IL-6 on steroidogenesis raises the question: how does IL-6 increase and decrease steroidogenesis in two tissues that are very similar? The tissue and cells used in this study were bovine zona fasciculata, zona reticularis, and human H295R cells. IL-6

was used in a dose and time dependent manner to study its effect on mRNA and protein levels of the nuclear factors DAX-1 and SF-1 and the enzymes involved in cortisol production, P450_{scc}, 17 α hydroxylase, 3 β HSD2, 21 hydroxylase, and 11 β hydroxylase in bovine zona fasciculata and human H295R adrenal cells; and mRNA and protein levels for the nuclear factors DAX-1 and SF-1 and the enzymes involved in androgen production P450_{scc}, 17 α hydroxylase, and 3 β HSD2 in bovine zona reticularis adrenal cells.

Materials and Methods

Media

DMEM/F12

The DMEM/F12 was purchased from GIBCO as a powder. Water and NaHCO₃ were added to the DMEM/F12 powder as outlined by the instructions that come with the powdered medium. The media was then filter sterilized using vacuum filters inside of a sterile hood. The medium was completed by addition of New Calf Serum (purchased from HyClone), penicillin-streptomycin (purchased from GIBCO), and insulin-transferrin-sodium selenite (ITS). For completion of 500 ml of DMEM/F12 medium 26.5 ml of New Calf serum, 4.25 ml of penicillin-streptomycin, and 530 μ l of ITS was added. Completion of medium was done only when needed and only 500ml were completed at one time. The complete and incomplete DMEM/F12 was stored in the refrigerator between 0° – 4° C.

RPMI

RPMI medium 1640 was purchased from GIBCO in powder form. The powder was mixed with sterile water and NaHCO₃ at room temperature. The solution was mixed using a magnetic stir bar until the powder was completely dissolved. The RPMI was then filter sterilized using a vacuum filter in a sterile hood. The RPMI 1640 medium was kept in a refrigerator at a temperature of 0° – 4° C. 500 ml of RPMI was completed by addition of fetal calf serum, horse serum, and 150 μ l of a mixed solution containing penicillin – G (Sigma), streptomycin (Sigma), gentamicin (Sigma), and amphotericin – B (Sigma). The mixture of antibiotics and antifungal

had a volume of 8 ml and contained 200 mg penicillin – G, 200 mg streptomycin, 15 mg amphotericin – B, and 400 mg gentamicin. 8 ml of distilled sterile water was added to the antibiotics and antifungal and then vortexed. RPMI was completed only when needed.

H295R cultured cells

H295R cells were incubated in DMEM/F12 complete medium. Five ml of DMEM/F12 complete was added to every 100 mm culture plate. The H295R cells were grown on the plates in an incubator set to 5% CO₂. Once the cells were 70-80% confluent they were used in the experiment.

Bovine Adrenal Fragments

Bovine adrenal glands were collected from the abattoir. The Adrenals were collected and placed in sterile 1X phosphate buffer solution (PBS) soon after the cow was killed. They were brought back to the laboratory where they were sliced and dissected into the different zones; ZF and ZR. Once the zones were separated they were cut into small fragments and used for an experiment the same day. Medium used for the bovine tissue treatments was RPMI incomplete containing IL-6 in picogram concentrations.

Time Course Experiments

Human Time Course

Cultured H295R cells were incubated with DMEM/F12 incomplete that contained 50 pg/ml of IL-6. The plates were placed in an incubator with 5% CO₂ for the duration of the treatment. Each plate of cells was incubated for a different amount of time. Incubation time ranged from 0 – 240 min. Treatments were started at different times so that they were all stopped at the same time.

Bovine Time Course

Bovine tissue fragments were incubated with serum free RPMI containing IL-6 for differing amounts of time in 15 ml centrifuge tubes. The tubes with the tissue were placed in a water bath and lightly shaken for the duration of the incubation at

37° C. Each sample contained ~200 mg of tissue. Times ranged from 0, containing no IL-6, to 240 min. The treatment that did not receive any IL-6 during incubation was incubated in serum free RPMI for the same amount of time as the longest treatment. The treatments were started at different times so as to end at the same time.

Dose Response experiments

Human Dose Response

Cultured H295R cells were incubated with DMEM/F12 serum free that contained IL-6. Each plate of cells received a different concentration of IL-6, 0 – 100 pg/ml. The duration of incubation was 60 min. The cultured cells were placed in an incubator set to 5% CO₂ and 37° C. Treatments were started and stopped together.

Bovine Dose Response

Bovine tissue fragments were incubated in 5 ml of serum free RPMI containing IL-6 in 15 ml centrifuge tubes. Each treatment received different concentrations of IL-6. The tubes with the tissue were capped and placed in a water bath at 37° C. They were lightly shaken for the duration of the incubation. Each time treatment contained ~200 mg of tissue. Concentrations of IL-6 ranged from 0 – 100 pg/ml. The treatments were started and stopped at the same time, 60 min.

RNA extraction

RNA extractions for both H295R cells and bovine tissue were performed using Trizol reagent and the protocol that comes with the Trizol (Sigma). For H295R cells the incubation medium was removed from the plates and Trizol was added, 2 ml per culture plate. Trizol reagent was allowed to sit on the plates for 2 min. The cells were then scraped off the plate using a sterile cell scraper (Sarstedt). The liquid from each plate was pipetted into a 15 ml centrifuge tube. The solution was incubated for 5 min to allow for complete lysis of the cells. Chloroform (0.5 ml chloroform per 1 ml of Trizol reagent) was then added to the solution. Upon addition of chloroform the tubes were capped and were vigorously shaken for 15 sec after which they were

placed in a Jouan centrifuge(company) and centrifuged at 3000 RPM for 20 min. The centrifuge was refrigerated to 4° C. When the centrifugation was completed, the top layer of the solution, the aqueous phase, was removed and put into a new 15 ml centrifuge tube. Isopropyl alcohol was added (0.5 ml of isopropyl alcohol per 1 ml of Trizol reagent) to this solution and incubated on ice for 10 min. After incubation the solution was centrifuged again at 3000 RPM for 10 min. During this centrifugation, an mRNA pellet formed at the bottom of the tube. The solution was removed and 75% ethanol was added (1ml 75% ethanol for every 1 ml of Trizol reagent) to wash the pellet. After adding the ethanol the tubes were lightly shaken by hand to dislodge the pellet from the bottom and allow for complete washing of the pellet. The 15 ml centrifuge tubes were centrifuged again for 5 min. The solution was removed from the tubes, and the pellet was allowed to dry for 5 – 10 min. Sterile nuclease free water was then added to the pellet. Amount of water varied depending on the size of the pellet. The 15 ml centrifuge tubes with the water and pellet were then placed in a water bath at 60° C until the pellet was dissolved.

Bovine tissue that was used for RNA extractions was centrifuged for 5 min at 300 RPM. The tissue formed a pellet and the solution was removed. The tissue was then removed from the centrifuge tube and placed into a homogenizer. 1 ml of Trizol reagent was added and the tissue homogenized until it was no longer in large fragments. This solution was poured into a new 15 ml centrifuge tube. The homogenizer was washed with 1 ml of Trizol, and this was added to the solution in the 15 ml centrifuge tube. This solution was incubated for 5 min to allow for complete lysis of the tissue. Chloroform was added to the 15 ml centrifuge tubes and the tubes were then centrifuged for 20 min at maximum RPM. After centrifuging the solution was in three phases: Aqueous (top, clear), interphase (middle, white layer), and organic (bottom, red). The aqueous phase was removed and placed into new 15 ml centrifuge tubes. Isopropyl alcohol was then added to the new 15 ml centrifuge tubes, which contained the aqueous phase, to precipitate the RNA. This solution was incubated on ice for 10 min and then centrifuged for 10 min. After centrifuging a white pellet was found on the bottom of the tube. The supernatant was removed from the tubes, and ethanol was added to wash the pellet. The tubes were then agitated to

dislodge the pellet so it was completely washed. The tubes were then centrifuged for 5 min. The ethanol was removed after centrifuging, and the pellet was air dried for 5 – 10 min. Nuclease free water was added to the dry pellet, 50 – 300 μ l depending on the size of the pellet, and then the tube was placed in a water bath at 60° C until the pellet dissolved.

RNA Quantification

NFW (150 μ l) was added to a 0.2 ml microcentrifuge tube as a standard. NFW (147 μ l) was added to all other tubes in which RNA would be quantified. 3 μ l of RNA solution was taken from each treatment group and added to the corresponding 0.2 ml tube with the 147 μ l of NFW (for a total of volume 150 μ l). The tubes were then vortexed and centrifuged. The solution was pipetted out of the 0.2 ml microcentrifuge tubes and into a cuvette, one treatment at a time, and quantified using a spectrometer (GeneQuant II Pharmacia Biotech, Cambridge, England). Once the concentrations were known, calculations were made to find out how many μ l of sample were needed to obtain 2 μ g of RNA for the RT (reverse transcriptase).

$2 \mu\text{g} / \text{concentration of sample} * 1000 \mu\text{l} / 1 \text{ml} = \mu\text{l of RNA sample to get } 2 \mu\text{g of RNA}$

RT- Reverse Transcription

A large mixture of reagents was made that contained 2 μ l 10x buffer, 1 μ l dNTP (deoxynucleotide triphosphates), 2 μ l DTT, 0.4 μ l random decamers, 1 μ l RNase out, and 1 μ l superscript II, the reverse transcriptase. Each of these values was multiplied by the number of treatments plus 1. From the grand mixture 7.4 μ l was pipetted into the appropriate number of 0.2 ml microcentrifuge tubes, one for each treatment. 2 μ g of RNA was added to the 0.2 μ l tubes (the volume of sample to obtain 2 μ g of RNA was calculated during the quantification). Nuclease free water was added to bring the total volume to 20 μ l. The tubes were then centrifuged, vortexed, and placed in a thermocycler (Gene Amp PCR system 2400 Perkin Elmer, Waltham, Massachusetts). The program was set to run for 60 min at 42° C.

Standard PCR- Polymerase chain reaction

A grand mixture was also made of the PCR reagents. The grand mixture was 39.975 μl of NFW, 5 μl of 10x buffer, 0.625 μl of 0.4 mM nucleotide triphosphate mixture (dNTP), 2 μl of 0.1 nmole/ μl mixture of sense and anti-sense of the primer which would be used, and 0.4 μl of Jstaq (250 units of DNA polymerase). These volumes were multiplied by the number of treatments plus 1. A different grand mixture was made for each primer used. 48 μl aliquots of the grand mixture were pipetted into 0.2 ml microcentrifuge tubes. 2 μl of the RT was then added making the total for each 0.2 ml tube 50 μl . The tubes were then vortexed, centrifuged, and placed in the thermocycler (Gene Amp PCR system 2400 Perkin Elmer, Waltham, Massachusetts). The thermocycler was programmed to cycle 35 – 55 times. The initial step denatured the cDNA. The temperature and time for this step was 95° C and 3 min. The next three temperatures were the temperatures that cycle 35 – 55 times. The first temperature of the cycle denatured the template and newly made complementary strand. This step required 95° C and different durations of time ranging from 20 sec to 30 sec depending on the length of the amplified sequence. The second temperature allowed for the annealing of the primer to the target cDNA. This step was set to different temperatures ranging from 55° – 58° C depending on the length of the primer and percent of purines and pyrimidines. The duration of this step ranged from 20 to 30sec. The third temperature was 72° C. This temperature was optimal for polymerase function and elongation of the amplified sequence. The duration of this step was changed depending on the length of the amplified sequence and ranged from 20 sec to 1 min. The final two temperatures of the thermocycler program were 72° C for 3 min for final elongation and 4° C for stopping inappropriate reactions until the PCR mixture was frozen (diagram 2).

Electrophoresis

The agarose gel was made by mixing 50 ml of 1x Tris-Acetate/EDTA electrophoresis buffer (1x TAE) with 0.5 g of agarose in a beaker and covered with plastic wrap. The solution was microwaved for 1 min. Then 2.5 μl of ethidium bromide was added to the solution. Once it was mixed, the solution was poured into

the gel tray and all bubbles were removed, the comb was then added to create the wells. The solution was then allowed to harden for 25 min.

10 μ l of the PCR solution was mixed with 2 μ l of loading dye and placed in a well of the gel. Also, 3 μ l of DNA ladder was placed into a well. The electrophoresis machine was set to 80 V and allowed to run for 25 min.

After 25 min the gel was removed from the tray and a picture was taken by reflecting ultraviolet light off of the gel. This was done using a computer camera (Alpha Innotech Corporation, Fluorchem 8900). The quantification was also done on the computer (Fluorchem 8900 software).

Real-Time PCR

(The converting of RNA to cDNA was done in the same manner as for standard PCR but was diluted with sterile nuclease free water by a factor of 50 for use in real time PCR).

Grand mixtures were made using a PCR supermix (2x mix contains 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), 50 U/ml iTaq DNA polymerase, 6 mM MgCl₂- iQ Sybergreen Supermix). The volume of supermix was 6.25 μ l times the number of reactions, plus 20% (# of reactions equals # treatments times the # of replicates). Primers (0.5 μ l times the # of replicates, plus 20%) and sterile nuclease free water (4.75 μ l times the # of replicates, plus 20%) were added to the PCR supermix in a 1.5 ml centrifuge tube. One 1.5 ml tube was prepared for each different primer. The 1.5 ml tubes were then vortexed and centrifuged. The mixture was then pipetted into 0.2 ml centrifuge tubes (total volume of grand mixture divided by the number of treatments), one 0.2 ml tube for each treatment. The RNA template was added to the 0.2 ml tubes at this point (0.167 μ l times the number of reactions, times 1.4). Each 0.2 ml tube was vortexed and centrifuged. The contents of the 0.2 ml tubes were pipetted into corresponding wells of a 96-well plate (12.5 μ l per well). The 96-well plate was then covered with sealing film and placed in the thermocycler (MyiQ Bio-Rad Laboratories, Hercules, California).

The thermocycler monitored and recorded the light emitted from the sample. The emitted light is produced from ultraviolet light when shone on SYBR green while the SYBR green reacts with the phosphate groups of DNA.

Melting temperatures of the products (fig.33) and gel electrophoresis of the products (pictures not shown) were used to identify the product and determine that only one product was being amplified by each primer used in real time PCR reactions.

Primer Design

Primers were designed using a primer design computer program from Invitrogen, Vector NTI, and ABI, Primer Express. The parameters were set to produce primers that would work with real-time PCR as well as standard PCR. Primers were made for DAX-1, SF-1, StAR, P450scc, 17 α hydroxylase, 3 β hydroxysteroid dehydrogenase type 2, 21 hydroxylase, and 11 β hydroxylase. Human and bovine specific primers were made.

Western Blotting

Protein extraction, H295R cells

After the completion of the treatment interval, the cells were rinsed in 1X PBS at room temperature. The 100 mm culture plates were then placed on ice and 0.6 ml of Radioimmunoprecipitation (RIPA) buffer was added. (100 ml of RIPA buffer contains 1 ml Nonidet P-40, 0.5 g Sodium Deoxycholate, 0.1 g SDS, and 1X PBS to bring the total volume to 100ml. At the time of use 10 μ l/ml of a 10 mg/ml concentration of Phenylmethyl sulfonyl fluoride, 30 μ l/ml of aprotinin (Sigma), and 10 μ l/ml of 100 mM sodium orthovanadate were added to the stock RIPA buffer). The culture plates were gently rocked for 15 min using a rocking platform from VWR Scientific Products (model 100). The culture plates were then scraped using a sterile cell scraper and the contents were pipetted into a 1.5 ml microcentrifuge tube. The solutions were then incubated on ice for 60 min. The tubes were then centrifuged at 10,000 G for 10 min at 4° C. The supernatant was transferred to another 1.5 ml

microcentrifuge tube and pellet discarded. Total concentration of protein was quantified using the Bradford assay.

Protein extraction, Bovine tissue

The bovine tissue, after treatment was complete, was centrifuged for 5 min at 300 RPM. The supernatant was discarded and the tissue was scraped into a homogenizer. 800 μ l of lysing buffer was added into the homogenizer (4 ml per gram of tissue, 200 mg of bovine tissue), and the tissue was homogenized until no large fragments remained. (Stock lysing buffer consisted of 0.79 g Tris-HCl, 4.6 g Mannitol, 0.21 g NaF 0.22 g Sodium pyrophosphate, 0.037g EDTA, 0.038 g EGTA, 1 ml Triton X100, 10 ml of glycerol, and distilled water to bring the total volume to 100 ml. At time of use 50 μ l of a 1 mM concentration of PMSF, 0.0154 g of dithiothreitol (Cleland's reagent, or DTT) (GIBCO), 0.0156 g of Benzamidine, and 0.1 mg of soybean Trypsin inhibitor (SIGMA) were added to the 100 ml stock solution). The solution was poured into a 1.5 ml microcentrifuge tube and incubated on ice for 60 min. The tubes were then centrifuged for 10 min at 10000 G. The solution was then frozen and stored in a freezer at -90° Celsius. Total concentration of protein was determined using the Bradford assay.

Electrophoresis

10 μ l of sample was loaded into a well of a polyacrylamide gel, one treatment per well, with a protein ladder also in a well alone. The gels (10%, BioRad) were electrophoresed for ~30 min at 200 V in a mini protean II (BioRad). The gels were removed; wells were cut away from the body of the gel, and placed in transfer buffer for 20 min with gentle rocking. After soaking the gels for 20 min they were placed in transfer cassettes. Transfer cassettes were assembled in a transfer cell (mini transblot cell, BioRad) and filled with transfer buffer. Proteins were transferred for 60 min at 100 mV. The amperes were adjusted to be above 200 mAmps. Once transfer was complete the nitrocellulose membrane (BioRad) was soaked in a 5% milk/PBST solution with gentle rocking for 1 hour to cover the membrane with a non-specific protein. After blocking nonspecific binding with milk the membrane was washed for

30 min. Washing started with PBST which was removed after 10 min and fresh PBST was put on. The second PBST solution was removed and PBS administered 20min into the wash. A final wash of PBS was applied 25 min after starting the washing process. At this point the primary antibody solution, 2% milk/PBST, was administered. The membrane was left, with the antibody solution, in a refrigerator overnight. The next day the antibody solution was poured off, and the membrane was washed with PBST and PBS for 30 min, PBST twice for 10 min and PBS twice for 5 min. The secondary antibody solution, 2% milk/PBST, was placed on the membrane for 1 hour. The antibody solution was removed from the membrane, and the membrane was washed with PBST and PBS for 30 min after. The washing consisted of PBST twice for 10 min each and PBS twice for 5 min each. At this point a picture was taken of the membrane. This was done by applying a detection solution (western blotting luminol reagent, Santa Cruz Biotechnology, Inc.) while in the darkroom, and placing a sheet of photo paper over the membranes to detect light emitted. The photo paper was processed in a developing machine and a computer was utilized to quantify the density of the bands (Fluorchem 8900, Alpha Innotech).

Antibodies

Antibodies for DAX-1, SF-1, StAR, P450scc, 17 α hydroxylase, 3 β hydroxysteroid dehydrogenase type 2, 21 hydroxylase, and 11 β hydroxylase were obtained from Santa Cruz. These antibodies were anti-human but were used for, and bound, the bovine proteins.

Results

Steroidogenic Factor-1/DAX-1

In the first series of experiments, the effects of IL-6 on the expression of the nuclear factors, Steroidogenic factor-1 (SF-1) and dosage sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (DAX-1) were determined. SF-1 causes activation of transcription for the steroidogenic genes whereas DAX-1 causes repression of these genes (22). To determine the effects of IL-6 on the mRNA and protein levels for the two transcription factors human H295R

cells and bovine ZF and ZR tissue were exposed to IL-6 in a time-dependent or dose-dependent manner. RT-PCR and western blot were utilized to quantify a change in the mRNA or protein.

Human H295R

In the human adrenocortical cell line H295R IL-6, in a time-dependent manner, decreased DAX-1 mRNA. A statistically significant decrease was first observed at 30 min which continued through 100 min (Fig. 1C). Similarly, IL-6 decreased significantly DAX-1 protein starting at 30 min and continuing through 120min of incubation (Fig. 2). In experiments utilizing different concentrations of IL-6, IL-6 decreased DAX-1 mRNA in a concentration-independent manner within the 60 min incubation interval. The decrease in DAX-1 mRNA was statistically significant with 1, 10, and 100 pg/ml IL-6 (Fig. 1A).

In contrast to DAX-1, IL-6 increased the expression of the mRNA and protein for SF-1. SF-1 mRNA increased significantly, 2.5 fold, at 100 min of incubation (Fig. 1C). Similarly, SF-1 protein level peaked at 120 min, 1.5 fold (Fig. 2A). SF-1 protein was significantly increased from 30 – 120 min of incubation. The concentration-related effects of IL-6 on SF-1 expression demonstrated an increasing pattern in which SF-1 increased significantly with 10 and 100 pg/ml IL-6, 1.8 and 1.5 fold respectively (fig. 1A).

To better clarify the relationship between DAX-1 and SF-1, the SF-1 to DAX-1 ratio was calculated. In the time course experiments, IL-6 exposure resulted in a significant increase in the ratio of SF-1:DAX-1 mRNA after 100 min of exposure to IL-6, 5 fold increase (Fig. 1D). The protein ratio increased significantly by 10 min and continued to increase to 30 min. Between 60 and 120 min the ratio dropped to a significant, 4 fold, increase (Fig. 2B). The ratio was statistically increased at all time intervals, 30, 60, and 120 min..

Bovine Zona Fasciculata

Similar to the H295R cells, IL-6 increased SF-1 protein and decreased DAX-1 protein in bovine ZF tissue. IL-6 decreased DAX-1 in a time-dependent manner with a decrease of 60% in DAX-1 protein at 120 min (Fig. 3A). The ZF tissue was very

sensitive to the IL-6-induced decrease in DAX-1. IL-6 concentrations of 1 pg/ml caused maximal inhibition of DAX-1 protein (Fig. 4A). More data is needed to determine if the change in protein is significant.

SF-1 protein increased in a time-dependent manner in bovine ZF tissue increasing within 30 min and reaching a 2.5 fold increase at 70 min (Fig. 3A). Dose-dependent treatments increased SF-1 protein an average of 2.5 fold with all three concentrations (Fig. 3A). More data is needed to determine if the change in protein is significant.

The protein ratio of SF-1:DAX-1 of ZF tissue treated in a time-dependent manner increased 5 fold at 120 min (Fig. 4B). The ratio of SF-1:DAX-1 protein in dose-dependent treatments increased with all concentrations with maximal effect being reached with 10 pg/ml (Fig. 4B). More data is needed to determine if the change in protein is significant.

Bovine Zona Reticularis

Because IL-6 inhibits the release of adrenal androgens from the bovine adrenal ZR, the effects of IL-6 on DAX-1 and SF-1 in ZR fragments were examined. To determine if the effect of IL-6 was time dependent, bovine ZR tissue was exposed to IL-6 (50 pg/ml) for time intervals from 10 – 120 min. IL-6 increased DAX-1 protein with a bi-nodal effect peaking at 30 min with a 2.5 fold increase which was down to a 1.5 fold increase at 70 min. By 120 min the protein was back up to a 2.25 fold increase (Fig. 5A). SF-1 protein decreased in a time dependent manner. SF-1 protein decreased 40% by 120 min (Fig. 5A). The ratio of SF-1:DAX-1 protein levels decreased 60% by 120 min (Fig. 5B). More data is needed to determine if the change in SF-1:DAX-1 protein is significant.

To determine if the effects of IL-6 on SF-1 and DAX-1 in ZR tissue were dose dependent, ZR fragments were incubated with different concentrations of IL-6 for 60 min. DAX-1 protein increased with 1 and 10 pg/ml IL-6, 20% (Fig. 6A). SF-1 protein decreased with all concentrations of IL-6 with 10 and 100 pg/ml causing the largest effect (Fig. 6A). The ratio of SF-1:DAX-1 protein showed a decrease of 40% with both 1 pg/ml and 10 pg/ml (Fig. 6B). More data is needed to determine if the change in protein is significant.

StAR

Because IL-6 affected the expression of DAX-1 and SF-1, nuclear factors that regulate steroidogenesis, increase cortisol release from the ZF and decrease adrenal androgen release from the ZR, the effects of IL-6 on the expression of StAR were determined. StAR transports cholesterol across the outer mitochondrial membrane where it can start the conversion to a steroid. This step is the rate limiting step of steroidogenesis.

Human H295R

In H295R cells, standard PCR demonstrated a significant, 2.5 fold, increase in StAR mRNA after 10 min exposure to IL-6 (Fig. 7). Likewise, StAR protein significantly increased 2 fold with 10 min of IL-6 exposure, at 30 min the increase was no longer significant. By 60 min StAR protein was significantly increased again and remained significantly increased through 120min (Fig. 8). To determine if the effect of IL-6 on StAR mRNA and protein was concentration dependent, H295R cells were incubated in medium containing different concentrations of IL-6 for 60 min. IL-6, 10 pg/ml, increased significantly, 2 fold, StAR mRNA levels. StAR mRNA was also significantly increased with 100 pg/ml (Fig 9).

Bovine Zona Fasciculata

Similar to H295R cells, IL-6 increased StAR mRNA and protein expression in ZF tissue in a time-dependent and concentration-independent manner. Real time PCR showed a significant change in mRNA at 30 min and 240 min of incubation, 2 fold at both time points (Fig. 10). The protein levels showed a 40% increase at 30 min which slowly decreased to 20% at 90 min (Fig. 16), more data is needed to determine if the change in protein is significant. Differing doses of IL-6 showed a significant 4 fold increase in mRNA with as little as 1 pg/ml, 10 pg/ml did not significantly increase in StAR (Fig. 11). Protein levels increased with 10 and 100 pg/ml causing a 1.5 and 2 fold increase respectively (Fig. 12), more data is needed to determine if the change in protein is significant.

Bovine Zona Reticularis

Bovine ZR cells treated with 50 pg/ml of IL-6 demonstrated a significant decrease in StAR mRNA levels of 60% within 30 min of incubation time and further decreased to 20% of the level of non-treated cells at 120 min (Fig. 13). StAR protein decreased 40% at 240 min, more data is needed to determine if the change is significant (Fig. 17). Bovine ZR tissue exposed to differing concentrations of IL-6 demonstrated a significant 30% decrease in mRNA levels with 10pg/ml IL-6 and maximal effect was reached with 100 pg/ml, 40% decrease (Fig. 14). StAR protein decreased significantly, and maximally, with 100 pg/ml IL-6, 45% (Fig. 15).

P450scc

P450scc is the first enzyme involved in the conversion of cholesterol to adrenal steroids. P450scc is found in the mitochondria and is the next rate limiting step, after translocation of cholesterol into the mitochondria by StAR. Transcription of P450scc has been demonstrated to be regulated by SF-1 and DAX-1. Therefore, the effects of IL-6 on P450scc protein and mRNA were determined using RT-PCR and western blot.

Human H295R

In H295R cells standard PCR demonstrated an increasing trend from 0 – 100 min. P450scc mRNA increased significantly at 60 min and was found to be not significantly elevated at 100 min (Fig. 7). Likewise, the protein levels for P450scc in H295R cells increased significantly within 10 min, and remained elevated, 2.2 fold, out to 120 min (Fig. 8).

To determine if the effects of IL-6 were dose dependent H295R cells were incubated in differing concentrations of IL-6 for 60 min. Standard PCR showed a significant increase in mRNA levels starting with 1 pg/ml of IL-6 (Fig. 9). Maximal effect was reached with 1 pg/ml, but 10 and 100 pg/ml also increased p450scc mRNA significantly.

Bovine Zona Fasciculata

Similar to H295R cells, IL-6 increased P450scc mRNA and protein in bovine ZF tissue. Real time PCR demonstrated a 4 fold increase at 140 min (Fig. 10). P450scc mRNA was significantly increased between 80 and 140 min, by 240 min the increase was no longer significant. Protein levels of P450scc detected by western blot showed an increase of 30% within 70 min and remained elevated, 20%, beyond 120 min (Fig. 18). More data is needed to determine if this effect on P450scc protein was significant.

The dose-dependent treatments of IL-6 elucidated the effect of IL-6 on P450scc mRNA and protein in bovine ZF fragments was not concentration dependent. Real time PCR demonstrated a larger effect with 1 pg/ml than the 10 pg/ml (Fig. 11). Both 1 and 10 pg/ml IL-6 exposure increased P450scc mRNA significantly, 2.4 and 2 fold respectively. Protein levels of P450scc in ZF fragments treated with differing concentrations of IL-6, likewise, showed an increasing trend with maximal effect being reached with 1 pg/ml of IL-6 (Fig. 12). More data is needed to determine if the effect on P450scc protein is significant.

Bovine Zona Reticularis

Because IL-6 decreased the SF-1:DAX-1 ratio in bovine ZR fragments, and the SF-1:DAX-1 ratio is thought to be a major factor of steroidogenesis by influencing the expression of steroidogenic enzymes, the effect of IL-6 on P450scc mRNA and protein was examined. To determine the effect of IL-6 on P450scc mRNA and protein in the bovine ZR, bovine adrenals were dissected, the ZR isolated, cut into fragments, and treated with IL-6. Real time PCR showed a significant decrease, 40%, in P450scc mRNA within 30 min of incubation (Fig. 13). At 240 min the mRNA level had plunged significantly to 20% of the control level. Western blot analysis demonstrated a similar trend (Fig. 21). Within 30 min of incubation the protein level for P450scc had dropped 80% and remained at this level beyond 240 min. More data is needed to determine if the change in protein is significant.

Because IL-6 was determined to have a time-dependent effect, it was also necessary to elucidate the concentration-dependent effect of IL-6 on P450_{scc} mRNA and protein in the bovine ZR. Real time PCR on cellular extracts from bovine ZR fragments demonstrated a dose response curve starting with 10 pg/ml of IL-6 and continuing to 100 pg/ml of IL-6 (Fig. 14). A significant 50% decrease was observed with 1 pg/ml and a 70% decrease with 100 pg/ml. Protein level detected by western blot demonstrated a significant decrease with 1, 10, and 100 pg/ml, 24%, 20%, and 21% respectively (Fig. 15).

17 α Hydroxylase

17 α hydroxylase is a unique enzyme because it is known to have two definitive catalytic functions. It converts pregnenolone to 17 hydroxy-pregnenolone and 17 hydroxy-pregnenolone to DHEA. The conversion of 17 hydroxy-pregnenolone to DHEA is augmented by cytochrome b₅ in the ZR. Therefore, DHEA is largely produced in the ZR.

Human H295R

In the human H295R cell line, standard PCR was used to quantify changes in mRNA for 17 α hydroxylase. Data demonstrated a significant increase in 17 α hydroxylase mRNA after 100 min of IL-6 exposure. An average increase of 3.5 was observed at 100 min (Fig. 7). Protein levels increased significantly at 30 min, reached 2 fold at 50 min, and remained significantly increased beyond 120 min (Fig. 8).

Bovine Zona Fasciculata

Similar to H295R cells, bovine ZF fragments increased 17 α hydroxylase mRNA and protein in response to IL-6 exposure. Real time PCR demonstrated a significant increase in 17 α hydroxylase mRNA starting at 30 min of incubation (Fig. 10). 17 α hydroxylase mRNA remained significantly elevated through 240 min. Protein levels increased 50% with 10 min of exposure to IL-6. 17 α hydroxylase protein demonstrated an increasing trend through 240 min (Fig. 20). More data is needed to determine if the change in protein was significant.

Because IL-6 increased 17 α hydroxylase mRNA and protein in a time-dependent manner, experiments were performed to clarify whether the mechanism was dose-dependent. Real time PCR determined the mechanism of IL-6 to be concentration independent. 1 and 10 pg/ml IL-6 significantly increased 17 α hydroxylase mRNA. Maximal effect was reached with 1 pg/ml (Fig. 11). Likewise, protein levels did not demonstrate a dose-dependent mechanism with 100 pg/ml IL-6 causing the same response as 1 and 10 pg/ml (Fig. 12). More data is needed to determine if the change in protein is significant.

Bovine Zona Reticularis

Because 17 α hydroxylase is necessary for adrenal androgen production, the effect of IL-6 on 17 α hydroxylase mRNA and protein was determined. Bovine ZR tissue treated with IL-6 for 120 min demonstrated an 80% decrease in 17 α hydroxylase mRNA. A significant decrease was seen starting at 30 min of incubation, 40% and continued through 120 min (Fig. 13). Protein levels decreased 80% within the first 10 min of exposure to IL-6 and remained decreased for 240 min (Fig. 21). More data is needed to determine if the change in protein is significant.

The next necessary step was to elucidate the concentration-dependent effects of IL-6 on the ZR. ZR tissue treated with different concentrations of IL-6 demonstrated a mechanism that was concentration independent within 60 min. Treatments of 5 – 100 pg/ml significantly decreased 17 α hydroxylase mRNA levels 25% and 40% respectively (Fig. 14). Protein levels decreased significantly, 30% – 45%, with exposure to IL-6 (Fig. 15). Maximal effect was reached with 100 pg/ml.

3- β Hydroxysteroid Dehydrogenase type 2

3 β hydroxysteroid dehydrogenase type 2 (3 β HSD2) is not a cytochrome containing enzyme like the other enzymes in this steroidogenic pathway. It removes a hydrogen atom from the hydroxyl group on the number 3 carbon of cyclopentanophenanthrene body converting it to a ketone. In this process the double bond between carbons 5 and 6 is hydrogenated and a new double bond is formed between carbons 4 and 5. Because 3 β HSD2 is known to be regulated by SF-1 and DAX-1 and is essential for cortisol and androgen production, H295R cells and bovine

tissue fragments were exposed to IL-6 to determine the effect on 3 β HSD2 mRNA and protein.

Human H295R

In the human cell line H295R, IL-6 increased 3 β HSD2 mRNA in a time-dependent manner. Standard PCR demonstrated a significant 100% increase in mRNA at 60 min which remained significantly elevated through 100 min (Fig. 7).

IL-6 was determined to act independently of concentration within 60 min on 3 β HSD2 mRNA and dependent on concentration for protein. 1 pg/ml IL-6 significantly increased 3 β HSD2 mRNA 3.5 fold, and 100 pg/ml 3 fold (Fig. 9). Maximal effect was reached with 1 pg/ml, and significant increases were observed with 1, 10, and 100 pg/ml.

Bovine Zona Fasciculata

Similar to H295R cells, bovine ZF treated with IL-6 demonstrated a time-dependent mechanism on 3 β HSD2 mRNA and protein. 50 pg/ml IL-6 increased 3 β HSD2 mRNA significantly at 30 and 80 min, quantified using real time RT-PCR (Fig. 9). At 240 the increase was 2 fold but was not significant. Protein levels, measured by western blot, peaked at a 0.6 fold increase at 90 min (Fig. 22). At 240 min the increase in 3 β HSD2 protein was 20%. More data is needed to determine if the change in protein was significant.

Experiments were performed to determine if the mechanism of IL-6 on 3 β HSD2 was concentration dependent. Bovine ZF tissue treated in a dose dependent manner showed the mechanism of IL-6 on 3 β HSD2 mRNA was concentration dependent. Real time PCR demonstrated a 3.5 fold increase of 3 β HSD2 mRNA with 1 pg/ml and a 4 fold increase with 10 pg/ml (Fig. 11). 3 β HSD2 protein increased 20% with 1 pg/ml (Fig. 12). Maximal effect was reached with 1 pg/ml. More data is needed to determine if the change in protein was significant.

Bovine Zona Reticularis

3 β HSD2 is more abundantly expressed in the ZF than the ZR (18). However, 3 β HSD2 is necessary for conversion of DHEA to other adrenal androgens. Bovine ZR tissue treated with 50 pg/ml of IL-6 for 120 min showed a significant 90%

decrease in 3 β HSD2 mRNA. 10 min exposure decreased significantly 3 β HSD2 mRNA 40%, all time points in between were significantly decreased (Fig. 13).

Different concentrations of IL-6 elucidated a significant 25% decrease with 1 pg/ml in 3 β HSD2 mRNA. Concentrations of 10 and 100 pg/ml caused a greater effect than 1 pg/ml, and only 10 and 100 pg/ml IL-6 caused a significant decrease compared to controls (Fig. 14). 3 β HSD 2 protein also decreased significantly with 1, 10, 100 pg/ml, 30%, 40%, and 50% respectively (Fig. 15).

21 Hydroxylase

21 hydroxylase is found in all zones of the adrenal cortex, ZG, ZF, and ZR (18). Located in the endoplasmic reticulum 21 hydroxylase oxidizes the 21 carbon of the cyclopentanophenanthrene steroid body by addition of a hydroxyl group. Because several of the enzymes involved in steroidogenesis are regulated by the SF-1:DAX-1 ratio the effect on 21 hydroxylase was examined.

Human H295R

Standard RT-PCR showed a significant increase in 21 hydroxylase mRNA starting after 100 min of exposure to IL-6, 3 fold (Fig. 7). 21 hydroxylase protein showed an increasing trend, however, it was not found to be significant at any time point (Fig. 8).

Bovine Zona Fasciculata

Similar to H295R cells, bovine ZF tissue increased 21 hydroxylase mRNA in response to IL-6. Real time PCR demonstrated a time-dependent effect of IL-6 on bovine ZF tissue. 21 hydroxylase mRNA increased significantly at 30 min, 3 fold (Fig. 10). The mRNA level peaked with a significant increase of 3.5 fold at 80 min and fell to a 2 fold increase at 240 min. After 80 min of incubation 21 hydroxylase mRNA was not significantly increased. Because IL-6 affected 21 hydroxylase in a time-dependent manner, experiments were performed to determine if the mechanism of IL-6 was dose-dependent. 1 pg/ml of IL-6 significantly increased, maximally, 3 fold 21 hydroxylase mRNA. The 10 pg/ml significantly increased 21 hydroxylase

mRNA 2 fold (Fig. 11). Therefore, IL-6 did not show a dose dependent mechanism at the mRNA level.

11 β Hydroxylase

11 β hydroxylase type 1 is found specifically in the ZF and ZR of the adrenal cortex (18). It is the last step in the production of cortisol and is found in the mitochondria. The first and last steps in the production of cortisol are performed in the intra-membrane space of the mitochondria. Experiments were performed to elucidate the mechanism of IL-6 on human cells and bovine ZF tissue on 11 β hydroxylase.

Human H295R

11 β hydroxylase mRNA increased significantly at 60 min and remained significantly elevated through 100 min, 3 and 2 fold respectively (Fig. 7). Likewise, 11 β hydroxylase protein increased significantly 3.2 fold after 60 min of exposure to IL-6. Protein levels remained significantly elevated through the 120 min incubation period (Fig. 8).

Bovine Zona Fasciculata

Analogous to H295R cells, bovine ZF increased 11 β hydroxylase in a time-dependent manner when exposed to IL-6. Real time PCR demonstrated a significant increase in 11 β hydroxylase mRNA of 4 fold at 30 min and remained significantly increased through 80 min. At 140 min a 2 fold increase was observed which increased to 3 fold between 140 – 240 min (Fig. 10). Only the 30 and 80 min time points were significantly increased compared to controls.

Concentration-dependent exposures to IL-6 demonstrated a concentration independent mechanism at the protein and mRNA level. Real time PCR demonstrated an increasing trend but no treatment was found to be significantly increased (Fig. 11). Likewise, 11 β hydroxylase protein demonstrated an increasing trend, but more data is needed to determine if the effect is significant (Fig. 12).

Discussion

Human and Bovine Zona Fasciculata

IL-6 is a cytokine released from numerous tissues including the immune system, exercising muscle, damaged tissue, and the adrenal cortex. IL-6 increases cortisol release from the human and bovine adrenal. The biochemical effects through which IL-6 increases cortisol production have not been identified. IL-6 binds to the IL-6 receptor which dimerizes and activates JAK proteins. JAK proteins then phosphorylate STAT proteins, which are nuclear factors that increase transcription of specific genes. Likewise, JAK proteins are capable of phosphorylating and activating other pathways which lead to increased transcription. Two of these looked at in this study were SF-1 and a transcription inhibitor DAX-1. It was found that the ratio of these to transcription factors was more important than the level of each by itself. Individual cells could have different basal expression of these factors. Therefore, the ratio of the two would be more important in determining the steroidogenic state of the cells/tissue. IL-6 has not previously been shown to decrease DAX-1. DAX-1 has been shown to be down regulated by ACTH in a mouse tumor cell which has the ZF phenotype (22). IL-6 uses a different receptor than ACTH and initially different cellular molecules. In both human and bovine ZF, DAX-1 protein and mRNA decreased rapidly, within the first 60 min. This decrease can not all be explained by reduced transcription and translation rate alone. Therefore, IL-6 could be activating a pathway which leads to the degradation of the DAX-1 protein. Likewise, SF-1 protein increases rapidly. The increase in SF-1 protein can not exclusively be explained by an increase in translation. IL-6 could be causing the inhibition of the degradation of SF-1 protein. Although an increase in SF-1 has been correlated with an increase in steroidogenesis, the change in the SF-1:DAX-1 ratio is not driving the increase in steroidogenesis. IL-6 has been shown to increase AP-1 proteins and the phosphorylation of these proteins (35). Also, STAT proteins have been shown to bind to the StAR promoter and increase StAR promoter gene activity (36). Therefore, the SF-1:DAX-1 ratio could be involved in fine adjustments of the level of steroidogenesis. The increase in SF-1:DAX-1 ratio does correlate with an increase in StAR, P450_{scc}, 17 α hydroxylase, 3 β HSD2, 21 hydroxylase, and 11 β hydroxylase at

the mRNA level. Therefore, SF-1 and other transcription factors could be forming a complex on the DNA at a promoter region. The promoter might be more active when the whole complex is present but still be active, to a lesser extent, with just a few components, ie. STAT, SF-1, CREB, and AP-1 proteins.

StAR mediates the rate limiting step of steroidogenesis. Therefore, a slight rise in StAR protein levels would cause a large increase in steroid production. In this study StAR protein levels increased but only 20 to 60 percent. This change was very small compared to changes in some of the enzymes. Likewise, the change in StAR protein was found to be bi-phasic, rising significantly at 10 min of incubation and being non-significant at 30 min. By 60 min StAR protein was significantly increased again. This rapid increase could cause rapid rise in cortisol in response to an acute stimuli. This effect seems to be regulated at the protein level only suggesting a change in the rate of inactivation/degradation of StAR.

StAR mRNA increased within 10 min in the human and 30 min in bovine ZF tissue and remained at this point for the remainder of incubation time. miRNA which was predicted to regulate StAR mRNA has been shown to increase in H295R cells when incubated in cAMP containing medium (37). This suggests the mRNA for StAR is closely regulated to allow for quick decrease in steroidogenesis when the stimulation is stopped.

SF-1 is required for the expression of most steroidogenic enzymes. In contrast, DAX-1 inhibits the expression of steroidogenic enzymes and StAR. Therefore, an increase in the SF-1:DAX-1 ratio could in part be driving the increase in expression of the steroidogenic enzymes and StAR in human and showing an increasing trend in the bovine ZF tissue.

Protein levels for the enzymes increased rapidly in the H295R experiments and the bovine model seems react in the same manner. This increase was too fast for do novo synthesis. Therefore, it is likely that IL-6 affects the protein level of these enzymes by decreasing the rate of degradation.

Bovine Zona Reticularis

The effect of IL-6 on the ZR is not well understood, but in previous studies IL-6 has been shown to decrease DHEA production in bovine ZR tissue (20). Because the human cell line used in this experiment is a tumor cell line, it was not possible to determine the effects of IL-6 on human adrenal zona reticularis cells. Therefore, only bovine ZR adrenal tissue was used. The ZR fragments were dissected out and incubated with IL-6 containing RPMI.

It was found that IL-6 has an opposite effect in the bovine ZR than it has in the bovine ZF. IL-6 decreased the mRNA and preliminary results show a decreasing trend in protein for: StAR, SF-1, P450_{scc}, 17 α hydroxylase, and 3 β HSD2. It also increased the mRNA and protein for DAX-1. Therefore, the SF-1:DAX-1 ratio in the ZR decreased which would cause a decrease in steroidogenesis.

The effect in the ZR is opposite of the effect in the ZF when stimulated with IL-6. This could be influenced by the suppressor of cytokine signaling (SOCS) proteins and other SOCS-box (a sequence of amino acids that interacts with ubiquitin ligase) containing proteins which are activated by cytokines. IL-6 has been shown to increase activation of SOCS-1 (32). Different levels of methylation of SOCS-1 gene were shown to cause different effects. High levels of methylation would cause the gene silencing and therefore high activation of STAT in response to IL-6. When the gene was void of methylation STAT activation was decreased in response to IL-6. Therefore, the SOCS-1 gene could be regulation differently in the ZF and ZR tissues.

Moreover, the decrease in protein levels of the steroidogenic enzymes and StAR could be influenced by the increase in SOCS protein activation. The SOCS proteins have been shown to increase the ubiquitination of cytokine receptors (33, 34) and the increase in SOCS-box containing proteins maybe regulating the enzyme levels and StAR in the ZR tissue.

Likewise, StAR protein and mRNA were decreased with IL-6 treatment in the bovine ZR. This effect was seen to be time-dependent but not dose-dependent for the 60 min of incubation. StAR is the rate limiting step of steroidogenesis. Therefore, a decrease in StAR would decrease the ability to produce steroids.

Similar to the human cell line and bovine ZF, the effect of IL-6 on the bovine ZR might be partly be controlled by the SF-1:DAX-1 ratio. DAX-1 is a transcription inhibitor which binds DNA and does not allow for the transcription to occur. Therefore, the increase in DAX-1 would decrease the transcription of the steroidogenic enzymes and StAR shift the state of the tissue away from steroidogenesis. The decrease in steroid production would cause a decrease in DHEA and other adrenal androgens produced by the adrenal ZR.

Protein levels of the enzymes, transcription factors, and StAR changed rapidly. This change in protein decreased as fast as the mRNA in many experiments. This decrease is hypothesized to be related to protein breakdown and not just a decrease in mRNA and translation. Therefore, IL-6 might decrease production of adrenal androgens by increasing processing of StAR and the steroidogenic enzymes in the bovine ZR.

Future Experiments

Continue with the western blots for the ZF and ZR tissue and H295R dose dependent treatments. More data is needed to determine if the preliminary results are valid.

To determine if SOCS proteins might be involved western blots will be done to find out basal and IL-6 stimulated levels in bovine ZF and ZR tissue. If the levels are significantly different between the two tissues then the amino acid sequence of the enzymes will be searched to find sequences that might interact with the SOCS-box and increase ubiquitination of the enzymes.

There have been many transcription factors shown to increase steroidogenesis in the adrenal. Which of these is involved with increase in steroidogenesis in the bovine and human adrenal cells needs to be determined. This can be tested by Gel mobility shift assays in which supershifts are involved. Transcription factors that will be examined include SF-1, AP-1 proteins, CREB, and STAT proteins.

Conclusion

The cytokine interleukin-6 is increased during physical and emotional stress. IL-6 increases cortisol production in human and bovine adrenal cells. A possible

mechanism by which IL-6 mediates this effect is through increasing the SF-1:DAX-1 ratio. The increase in this ratio would increase transcription of steroidogenic enzymes and StAR. StAR is part of the rate limiting step in steroidogenesis. Therefore, an increase in StAR would cause an increase in steroid production. Likewise, an increase in all the enzymes involved in cortisol production would increase steroid production. This research supports this hypothesis because IL-6 increased SF-1, known to be involved in transcription of steroidogenic genes in other tissues, and decreased DAX-1, a transcription repressor. In contrast, the effect of IL-6 on the bovine ZR tissue is opposite that of human H295R cells and bovine ZF tissue. IL-6 decreases P450_{scc}, 17 α hydroxylase, 3 β HSD2, 21 hydroxylase, 11 β hydroxylase, and StAR at the mRNA and protein levels. This effect may also be mediated by the SF-1:DAX-1 ratio. IL-6 increased DAX-1 and decreased SF-1 in this study. Therefore, IL-6 decreased the transcription activator, SF-1, and increased the repressor, DAX-1. Consequently, during times of physical and emotional stress, when IL-6 is elevated, cortisol would increase and androgen production would decrease. This change in adrenal steroidogenesis would cause a suppression of the immune system, increased anxiety, increased blood pressure, changes in metabolism, and decreased bone formation. Therefore, by knowing how IL-6 regulates adrenal steroidogenesis better treatments might be produced for patients suffering from diseases which involve high levels of inflammation and stress.

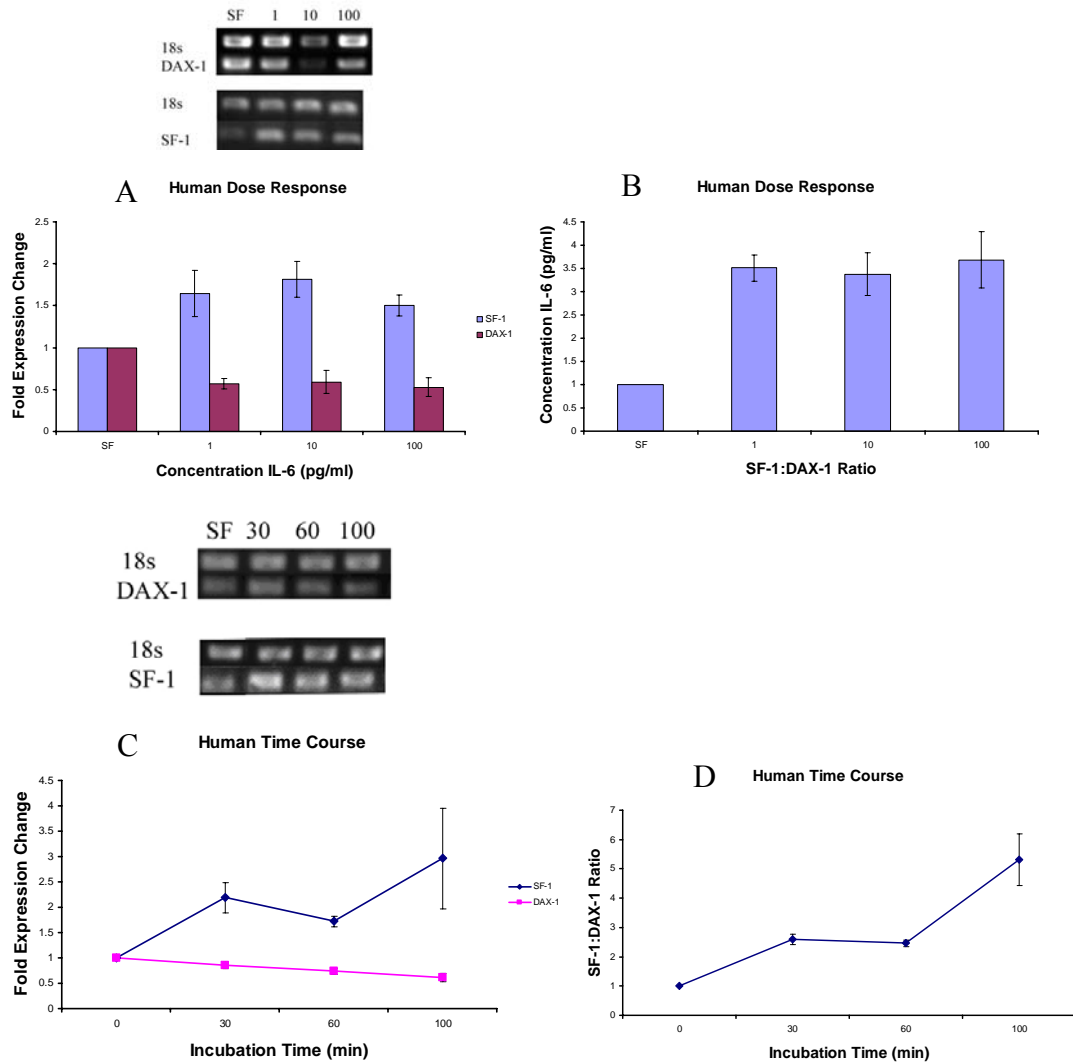


Fig. 1: Human Time and Dose Response SF-1 DAX-1, Standard PCR

Fig. 1A: Graph of SF-1 and DAX-1 in response to different concentrations of IL-6. SF-1 increased 1, 10, and 100 pg/ml IL-6. DAX-1 decreased significantly with 1, 10, and 100 pg/ml IL-6. Fig. 1B: Graph of the ratio of SF-1:DAX-1 in response to different concentrations of IL-6. The SF-1:DAX-1 ratio increase significantly with 1, 10, and 100 pg/ml Fig. 1C: Graph of SF-1 and DAX-1 mRNA in response to IL-6 dependent on time of incubation. SF-1 mRNA increased significantly after 120 min of incubation with IL-6. DAX-1 mRNA decreased significantly by 30 min and remained significantly decreased through 120 min. Fig. 1D: SF-1:DAX-1 mRNA ratio in response to IL-6 and dependent on time of incubation. The SF-1:DAX-1 ratio significantly increased at min after 100 min of incubation. Data was analyzed using bonferroni test and a P value of 0.05. Data consists of 6 samples.

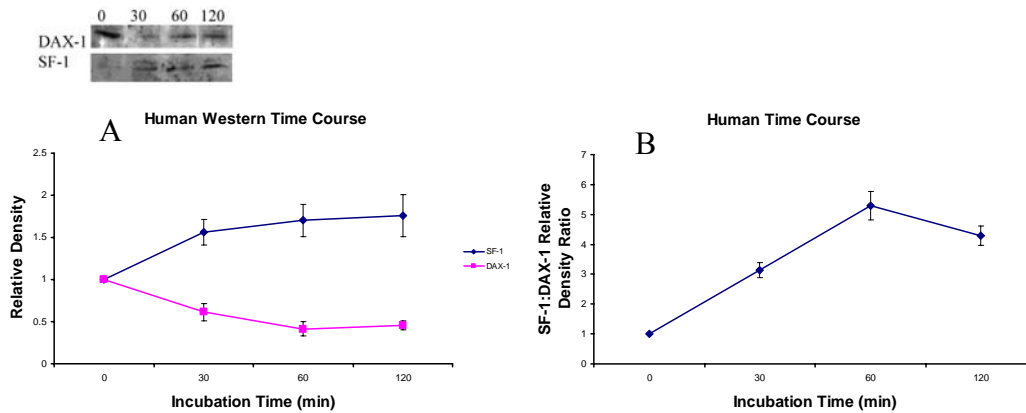


Fig. 2: Human Time Course SF-1 DAX-1, Western Blot

Fig. 2A: Graph of the change in protein dependent on time of incubation with IL-6. SF-1 protein increased significantly after 30 min of incubation time. SF-1 protein remained significantly increased through 120 min. DAX-1 protein decreased significantly after 30 min of incubation time and remained significantly decreased through 120 min. Fig. 2B: graph of the change in SF-1:DAX-1 protein ratio. The SF-1:DAX-1 protein ratio increased significantly by 30 min and remained significantly increased through 120 min.

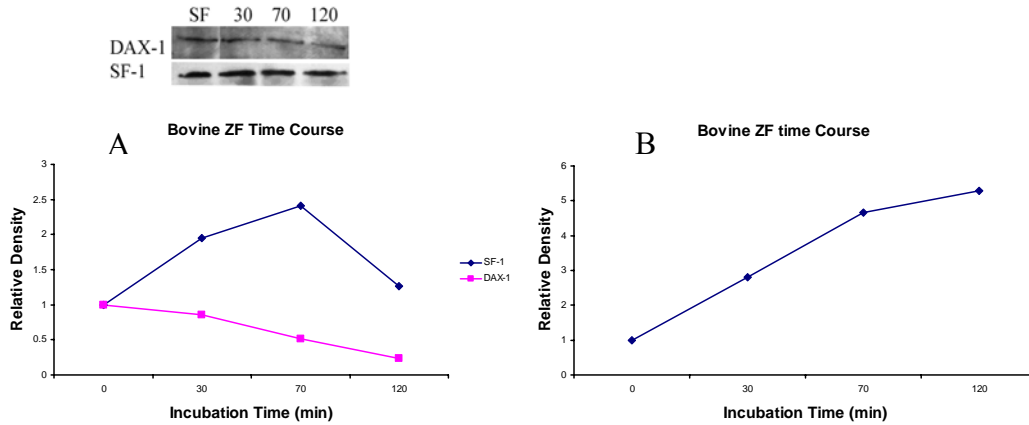


Fig. 3: Bovine ZF Time Course SF-1 DAX-1, Western Blot

Fig. 3A: Graph of the change in SF-1 and DAX-1 protein dependent incubation time with IL-6. Fig. 3B: Graph of the ratio of SF-1:DAX-1 protein. More data is needed to determine if the change is significant.

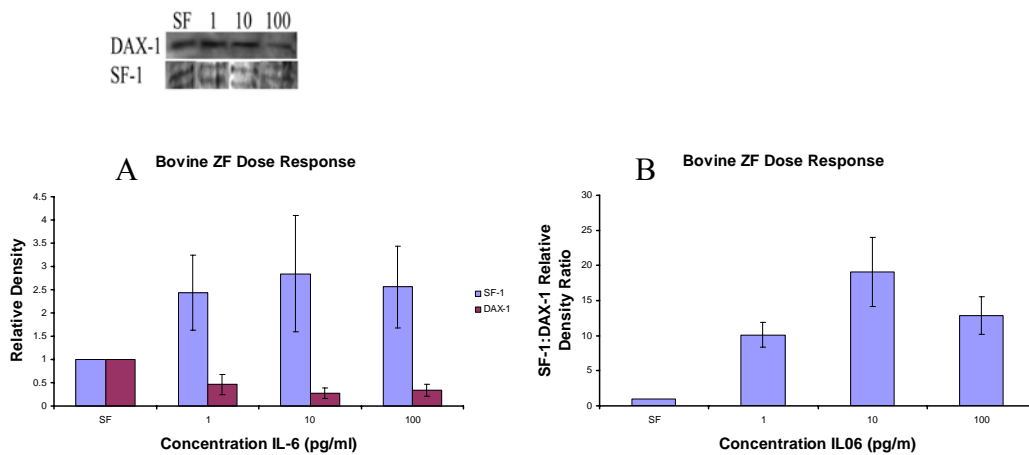


Fig. 4: Bovine ZF Dose Response SF-1 DAX-1, Western Blot

Fig. 4A: Graph of the change in SF-1 and DAX-1 protein dependent on the concentration of IL-6. Fig. 4B: is a graph of the ratio of SF-1:DAX-1 protein dependent on the concentration of IL-6. More data is needed to determine if the change is significant.

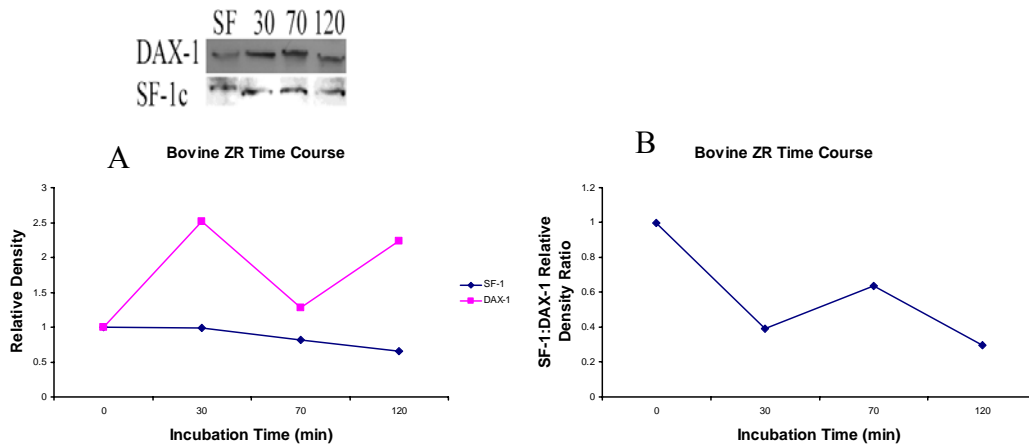


Fig. 5: Bovine ZR SF-1:DAX-1 Time Course, Western Blot

Fig. 5A: is a graph of the change in SF-1 and DAX-1 protein in response to different time of incubation with IL-6. Fig. 5B: Graph of the change in the SF-1:DAX-1 ratio. More data is needed to determine if the change in protein is significant.

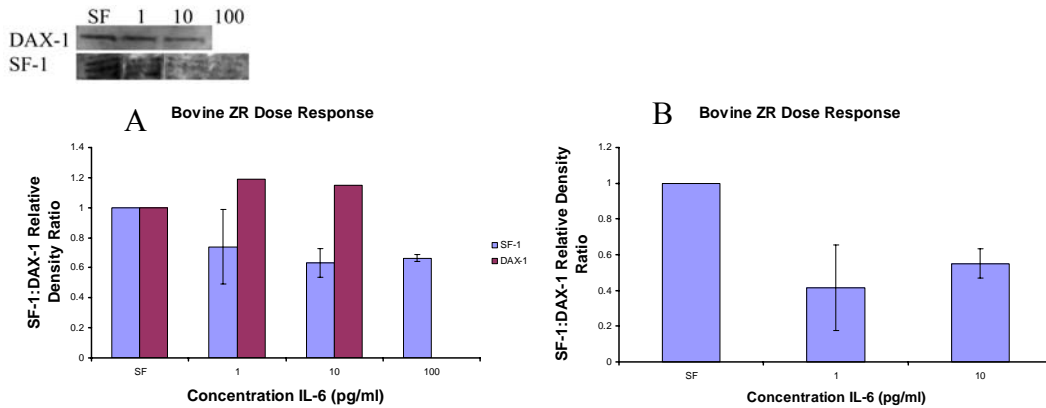


Fig. 6: Bovine ZR SF-1:DAX-1 Dose Response, Western Blot

Fig. 6A: Graph of the change in SF-1 and DAX-1 protein in response to different concentration of IL-6. Fig. 6B: Graph of the change in the SF-1:DAX-1 ratio. More data is needed to determine if the change in protein is significant.

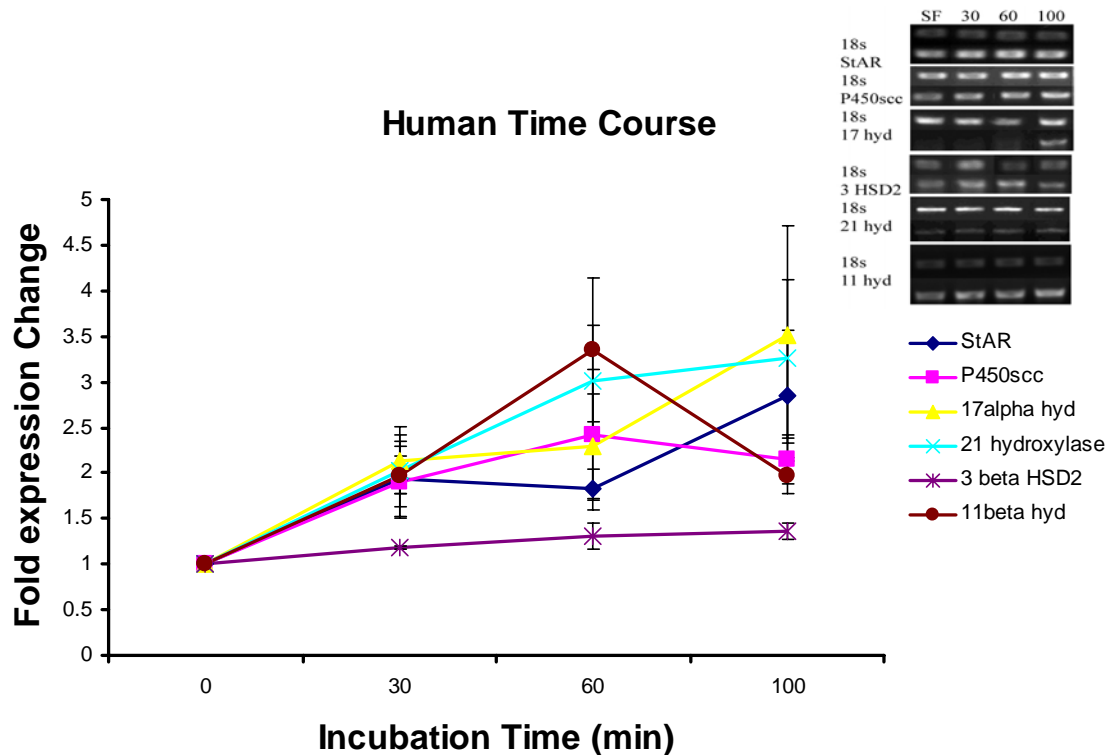


Fig. 7: Human Time Course StAR and enzymes, Standard PCR

Graph of the change in mRNA for StAR, P450scc, 17 α hydroxylase, 3 β HSD2, 21 hydroxylase, and 11 β hydroxylase in human H295R cells incubated in medium containing IL-6. StAR mRNA was significantly increased by 100 min. P450scc mRNA significantly increased by 60 min and was not significantly increased by 100 min. 17 α hydroxylase mRNA increased significantly by 100 min. 3 β HSD2 was significantly increased by 60 min and remained significantly increased through 100 min. 21 hydroxylase mRNA was significantly increased at 100 min of incubation. 11 β hydroxylase increased significantly by 60 min and remained significantly elevated through 100 min. Data was analyzed using the bonferroni test and a P value of 0.05. Data consist of 6 samples.

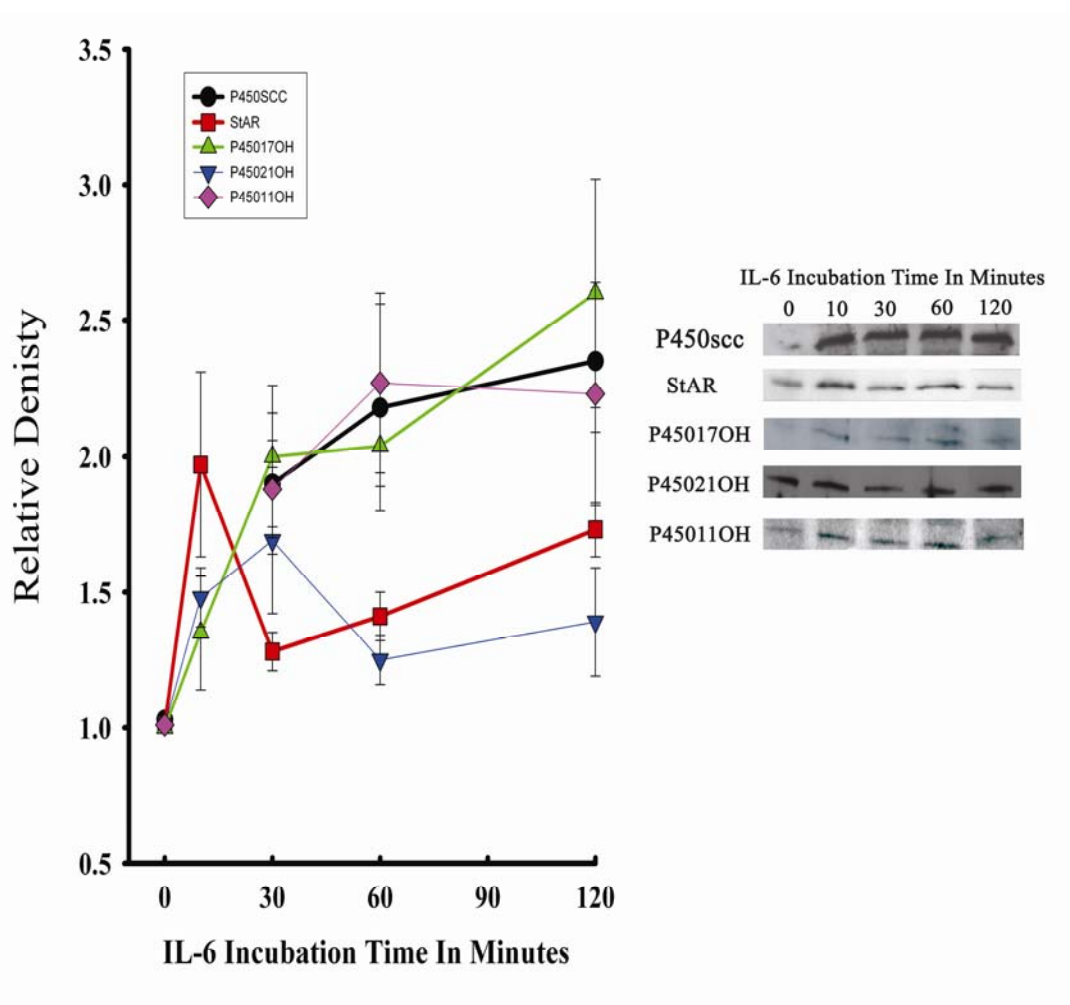


Fig. 8: Human Time Course StAR and enzymes, Western Blot

Graph of StAR, P450scc, 17 α hydroxylase, 21 hydroxylase, and 11 β hydroxylase protein levels in response to IL-6 and dependent on time of incubation. StAR protein increased significantly at 10 min of incubation. By 30 min StAR was no longer significantly increased. At 60 min StAR was increased significantly and remained through 120 min. P450scc increased significantly by 60 min of incubation and remained significantly elevated through 120 min. 17 α hydroxylase protein increased significantly by 30 min and remained significantly increased through 120min. 21 hydroxylase protein remained constant throughout the incubation period. No time point was found to be significantly increased. 11 β hydroxylase protein increased significantly by 60 min and remained significantly increased through 120min. Data was analyzed using bonferroni test with a P value of 0.05. Data consists of 6 samples.

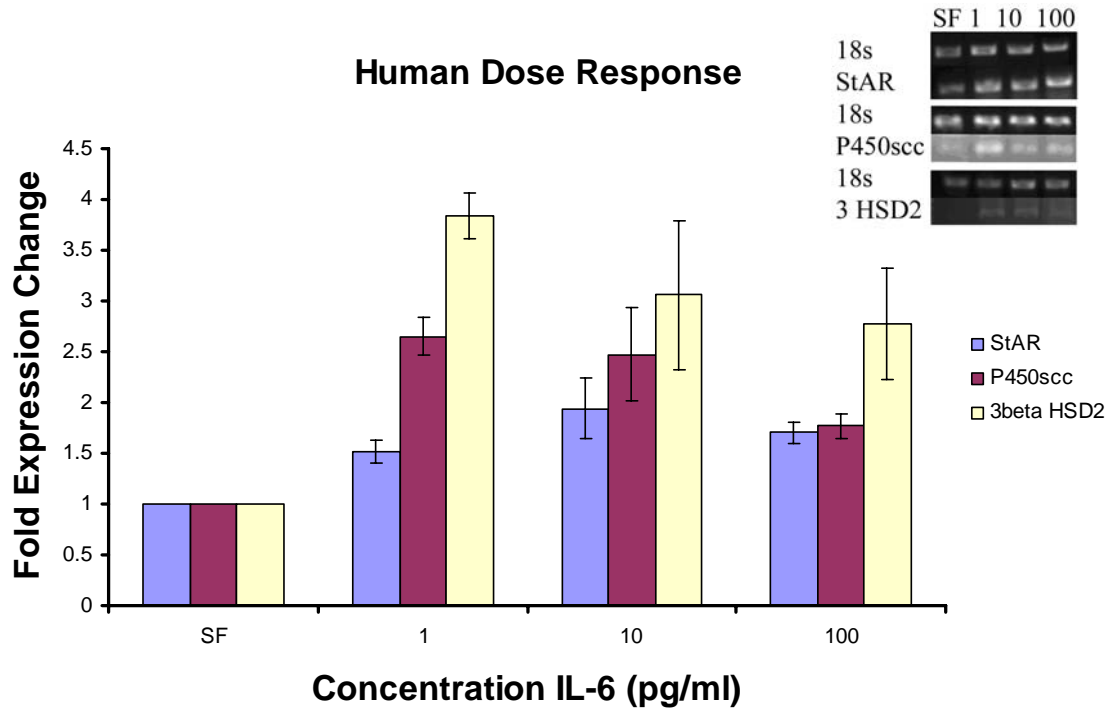


Fig. 9: Human Dose Response StAR and enzymes, Standard PCR

Graph of mRNA change for StAR, P450scc, and 3β HSD2 in response to different concentrations of IL-6. StAR mRNA increased significantly with 10 and 100 pg/ml. P450scc mRNA increased significantly with 1, 10, and 100 pg/ml. 3β HSD2 mRNA increased significantly with 1, 10, and 100 pg/ml. Data was analyzed using the bonferroni test with a P value of 0.05. Data consists of 6 samples.

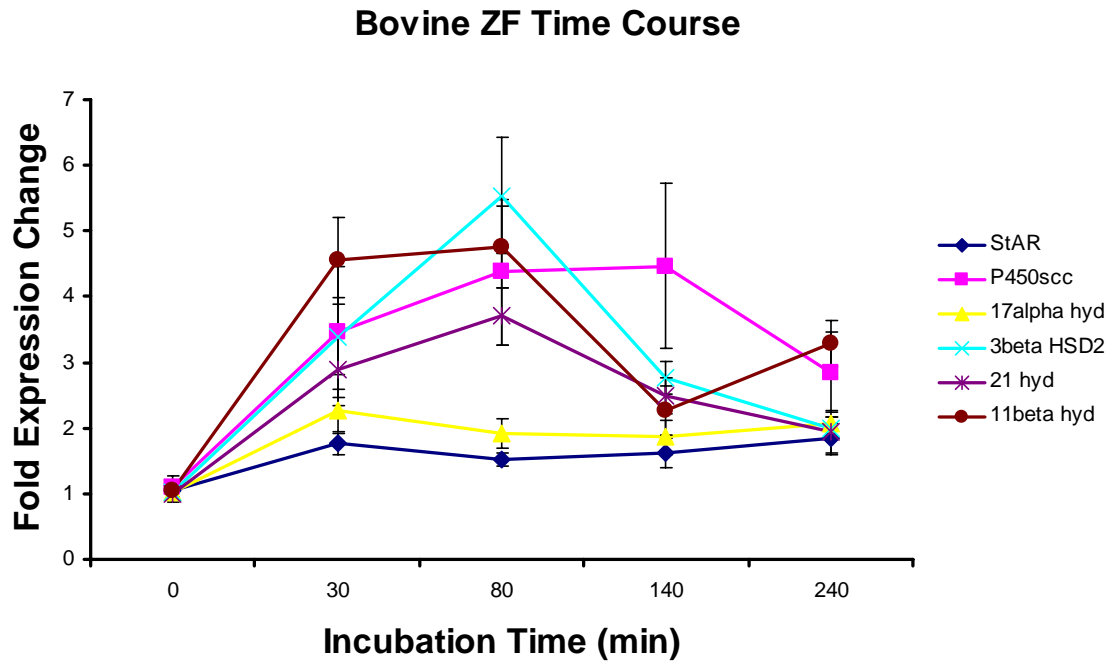


Fig. 10: Bovine ZF Time Course StAR and enzymes, Real Time PCR
 Graph of the change in mRNA for StAR, P450scc, 17 α hydroxylase, 3 β HSD2, 21 hydroxylase, and 11 β hydroxylase in bovine ZF tissue incubated with IL-6 containing medium. StAR increased significantly at 30 min. At 80 and 140 min the change was not significant. By 240 min StAR mRNA was again significantly increased. P450scc mRNA was significantly increased by 80 min and remained elevated through 140 min. By 240 min P450scc mRNA was not significantly increased. 17 α hydroxylase mRNA increased significantly by 30 min and remained significantly elevated through 240 min. 3 β HSD2 mRNA increased significantly by 30 min and remained through 80 min. By 140 min 3 β HSD2 mRNA was no longer significantly increased. 21 hydroxylase increased significantly at 30 and 80 min. At 140 min of incubation 21 hydroxylase mRNA was not significantly increased. 11 β hydroxylase increased significantly only at 30 and 80 min of incubation. Data was analyzed using the bonferroni test and a P valued of 0.05. Data consist of 8 samples.

Bovine ZF Dose Response

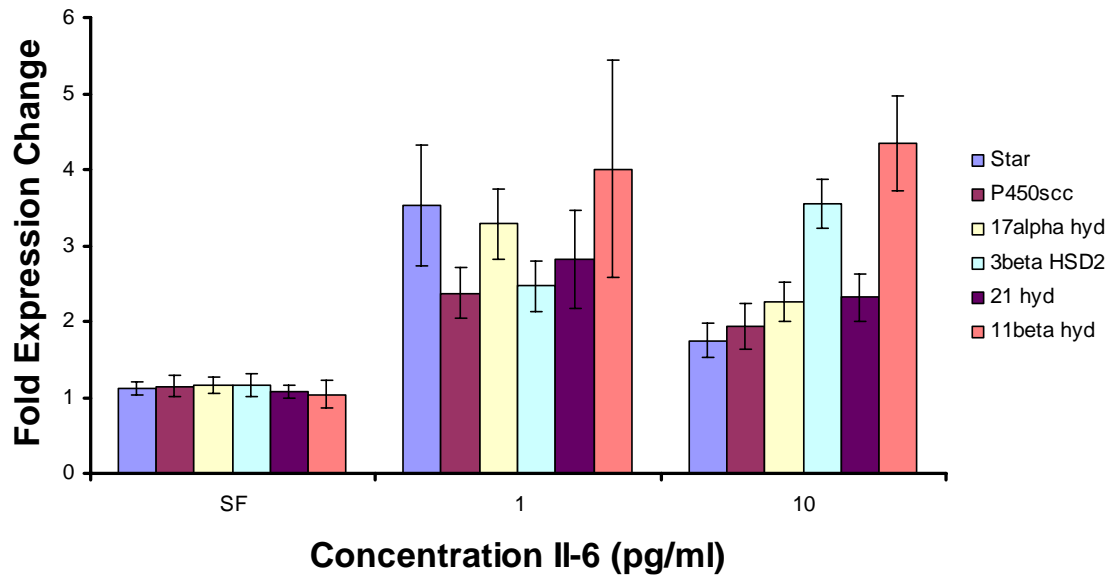


Fig. 11: Bovine ZF Dose Response StAR and enzymes, Real Time PCR

Graph of the change in mRNA for StAR, P450scc, 17 α hydroxylase, 3 β HSD2, 21 hydroxylase, and 11 β hydroxylase. StAR mRNA was significantly increased with 1 but not 10 pg/ml IL-6. P450scc mRNA was significantly increased with 1 and 10 pg/ml IL-6. 17 α hydroxylase was significantly increased by 1 and 10 pg/ml IL-6. 3 β HSD2 mRNA significantly increased with both 1 and 10 pg/ml. 21 hydroxylase mRNA increased significantly with both 1 and 10 pg/ml. 11 β hydroxylase mRNA did not significantly increase with either concentration of IL-6. Data was analyzed using the bonferroni test and a P value of 0.05. Data consists of 6 samples.

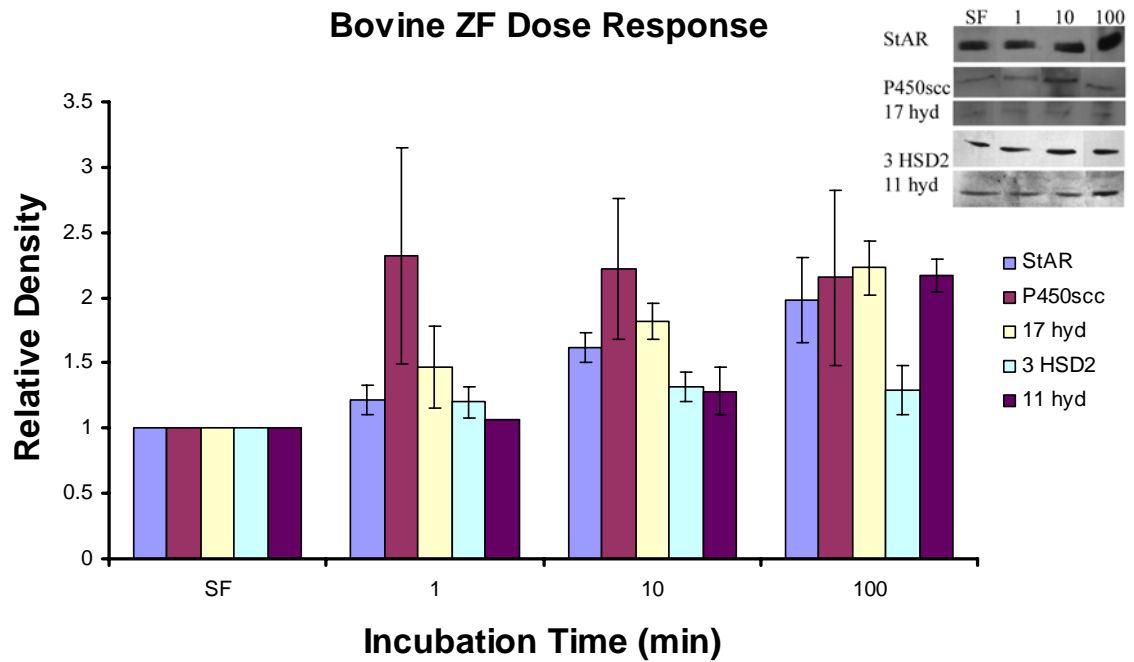


Fig. 12: Bovine ZF Dose Response StAR and enzymes, Western Blot
 Graph of the change in StAR, P450scc, 17 α hydroxylase, 3 β HSD2, and 11 β hydroxylase in response to different concentrations of IL-6. More data is needed to determine if the changes are significant.

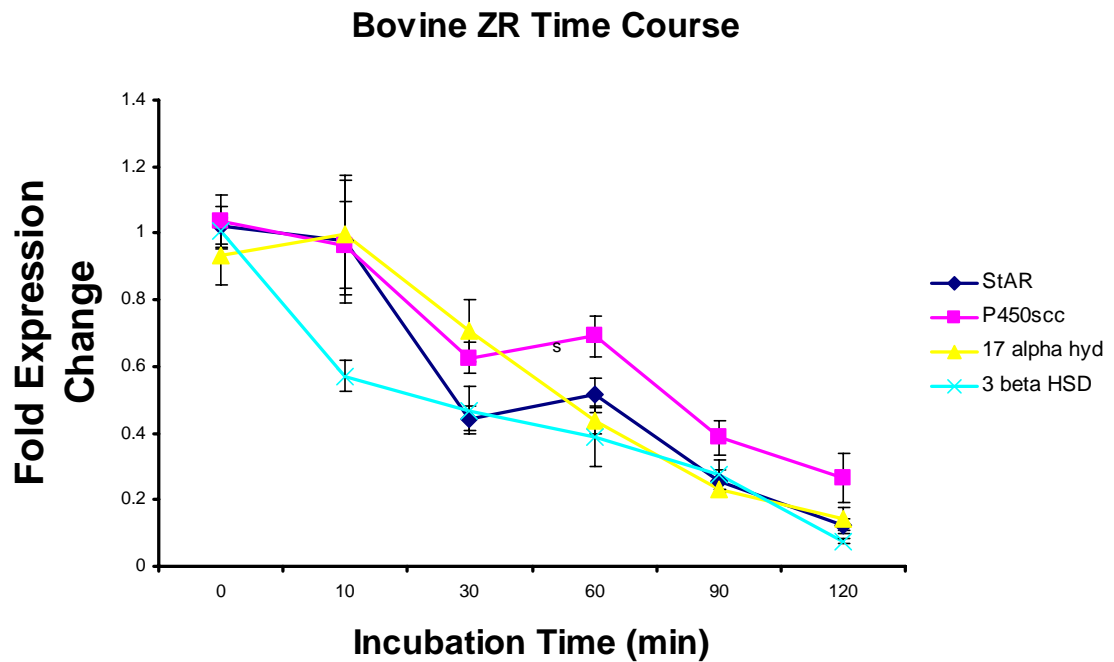


Fig. 13: Bovine ZR Time Course StAR and enzymes, Real time PCR
 Graph of the change in mRNA for StAR, P450scc, 17 α hydroxylase, and 3 β HSD2 in bovine ZR tissue incubated with IL-6 containing medium. StAR mRNA decreased significantly by 30 min and remained significantly decreased through 120 min. P450scc mRNA also decreased significantly by 30 min and remained significantly decreased through 120 min. 17 α hydroxylase mRNA decreased significantly by 60 min and remained significantly decreased through 120 min. 3 β HSD2 mRNA decreased significantly within the first 10 min of incubation and remained significantly decreased through 120 min. Data was analyzed using the bonferroni test and a P value of 0.05. Data consist of 12 samples.

Bovine ZR Dose Response

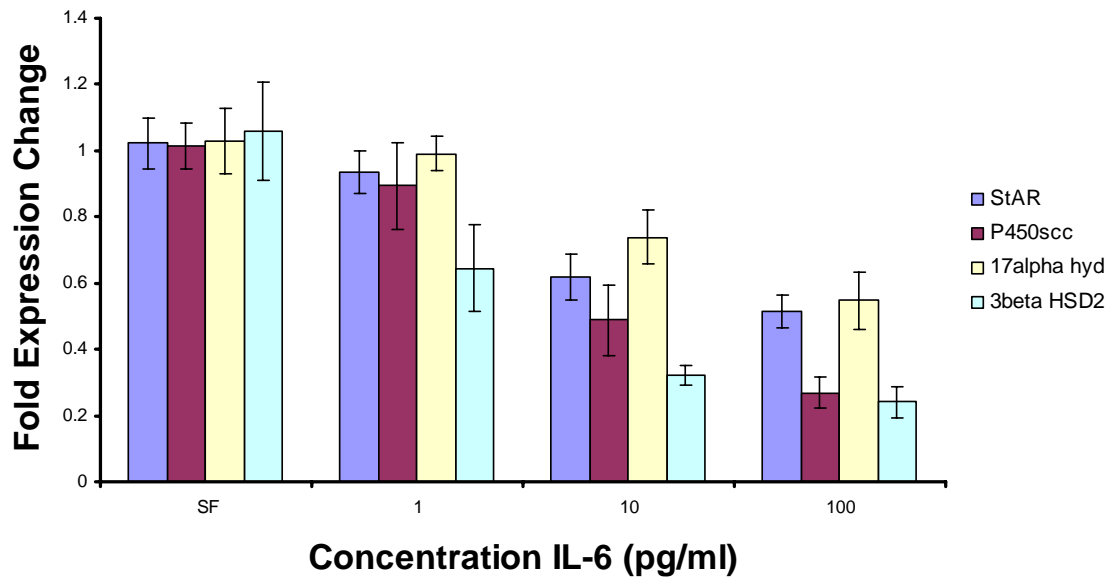


Fig. 14: Bovine ZR Dose Response StAR and enzymes, Real Time PCR

Graph of the change in mRNA for StAR, P450scc, 17 α hydroxylase, and 3 β HSD2 in bovine ZR tissue incubated in medium containing IL-6. StAR decreased significantly in 10 and 100 pg/ml and maximal effect being reached with 100 pg/ml. P450scc also decreased significantly with 10 and 100 pg/ml. The maximal decreased in P450scc mRNA was reached with 100 pg/ml. 17 α hydroxylase mRNA decrease significantly with both 10 and 100 pg/ml with maximal effect reached with 100 pg/ml. 3 β HSD2 mRNA decreased significantly with 1, 10, and 100 pg/ml. The maximal effect was reached with 100 pg/ml. Data was analyzed using a bonferroni test and a P value of 0.05. Data consist of 8 samples.

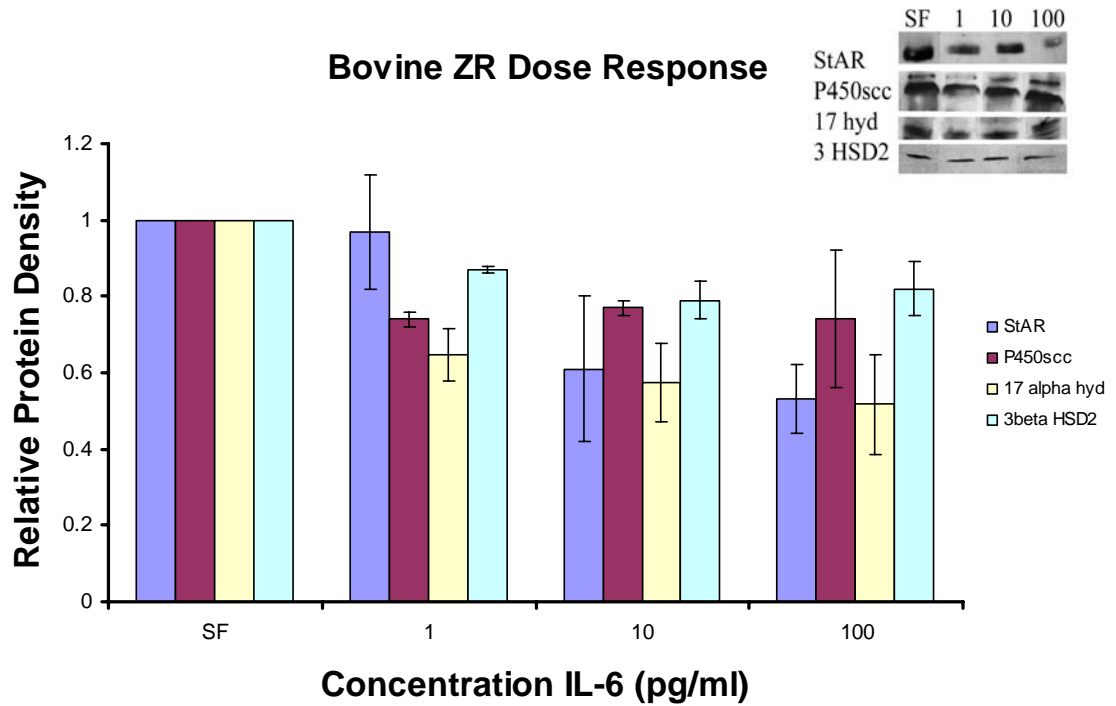


Fig. 15: Bovine ZR Dose Response StAR and enzymes, Western Blot

Graph of the change in protein, dependent on the concentration of IL-6, for StAR, P450scc, 17 α hydroxylase, 3 β HSD2, and 11 β hydroxylase. StAR protein decreased significantly with 100 pg/ml IL-6. P450scc decreased significantly with 1,10, and 100 pg/ml. 17 α hydroxylase decreased significantly with 1, 10, and 100 pg/ml IL-6. 3 β HSD2 protein significantly decreased with 1, 10, and 100 pg/ml IL-6. Data was analyzed using bonferroni test and a P value of 0.05. Data consist of 3 samples.

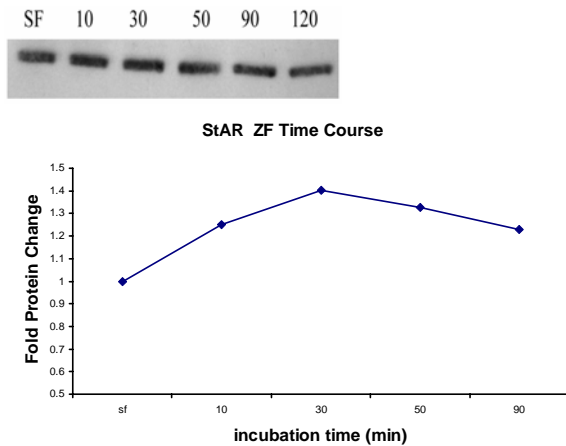


Figure 16: Bovine ZF StAR Western Blot

The Bovine ZF tissue was treated with IL-6 containing serum free RPMI for time intervals ranging from 10 – 90 min. The protein was then extracted from the tissue. Western blot analysis was used to determine the effects of IL-6 as a function of time on the StAR protein level. More data is needed to determine if the changes are significant.

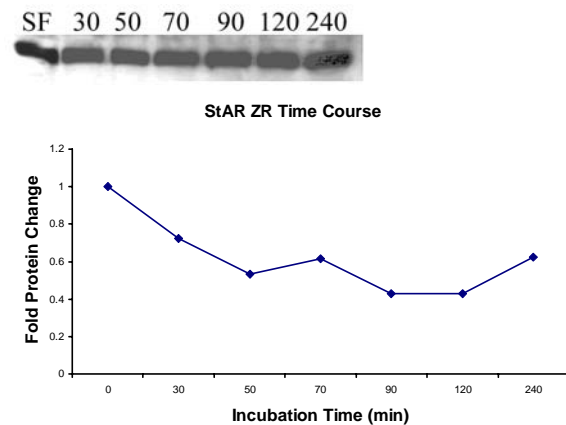


Figure 17: Bovine ZR StAR Western Blot

The Bovine ZR tissue was treated with IL-6 containing serum free RPMI for time intervals ranging from 30 – 240 min. The protein was then extracted from the tissue. Western blot analysis was used to determine the effects of IL-6 as a function of time on the StAR protein level. More data is needed to determine if the changes are significant.

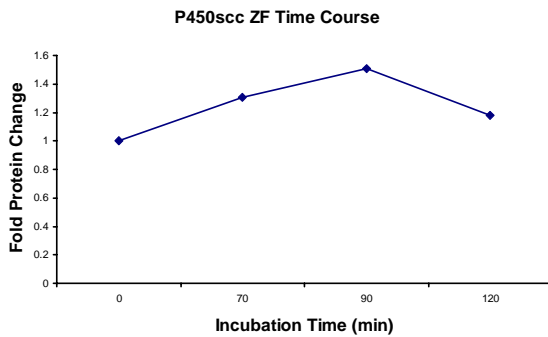


Figure 18: Bovine ZF P450scc Western Blot

The Bovine ZF tissue was treated with IL-6, 50 pg/ml, containing serum free RPMI for different time intervals. The protein was then extracted from the tissue. Western analysis was used to determine the effects of IL-6 as a function of time on the P450scc protein level. More data is needed to determine if the changes are significant.

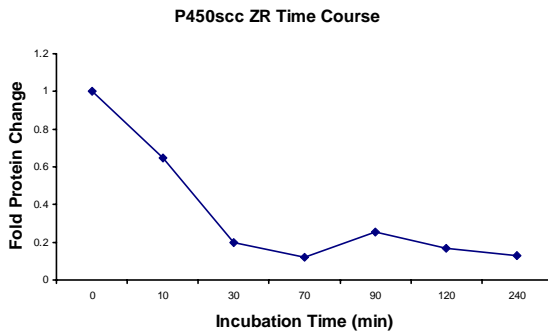
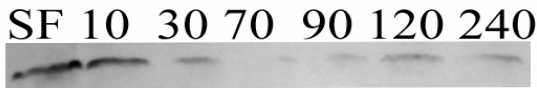
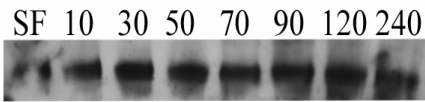


Figure 19: Bovine ZR P450scc Western Blot

The Bovine ZR tissue was treated with IL-6 containing serum free RPMI for time intervals ranging from 10 – 240 min. The protein was then extracted from the tissue. Western blot analysis was used to determine the effects of IL-6 as a function of time on the P450scc protein level. More data is needed to determine if the changes are significant.



17 alpha Hydroxylase ZF Time Course

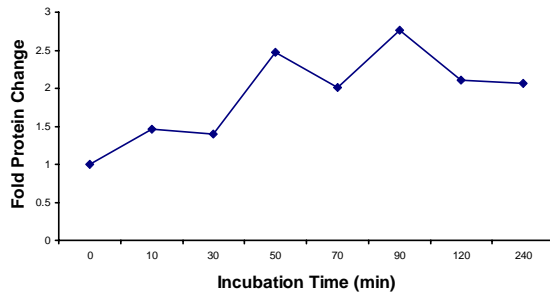


Figure 20: Bovine 17 α Hydroxylase Western Blot

Bovine ZF tissue was incubated in serum free RPMI containing 50 pg/ml IL-6 for different time intervals. The tissue was lysed, and the protein was extracted. The protein was then used for western blot analysis of the IL-6 effects on 17 α hydroxylase protein level as a function of time. More data is needed to determine if the changes are significant.



17 alpha Hydroxylase ZR Time Course

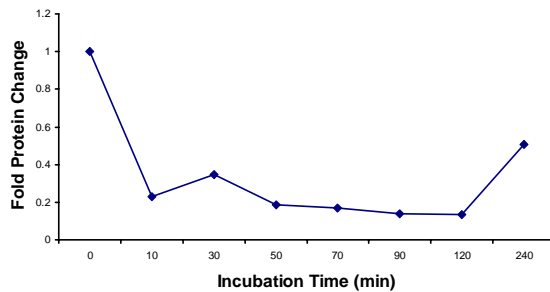
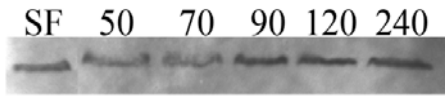


Figure 21: Bovine ZR 17 α Hydroxylase Western Blot

Bovine ZR tissue was incubated in serum free medium containing 50 pg/ml IL-6 for different time intervals. The tissue was lysed, and the protein was extracted. The protein was then used for western blot analysis to determine the effects of IL-6 as a function of time on 17 α Hydroxylase protein. More data is needed to determine if the changes are significant.



3 beta HSD2 ZF Time Course

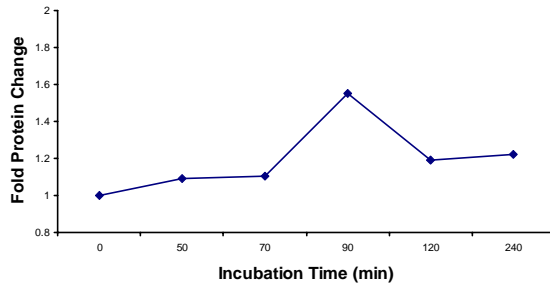


Figure 22: Bovine ZF 3 β HSD2 Western Blot

Bovine ZF Tissue was incubated in serum free RPMI containing 50 pg/ml IL-6 for different time intervals. The tissue was lysed, and the protein was extracted. The protein was then used for western blot analysis of the IL-6 effects on 3 β HSD2 protein level as a function of time. More data is needed to determine if the changes are significant.

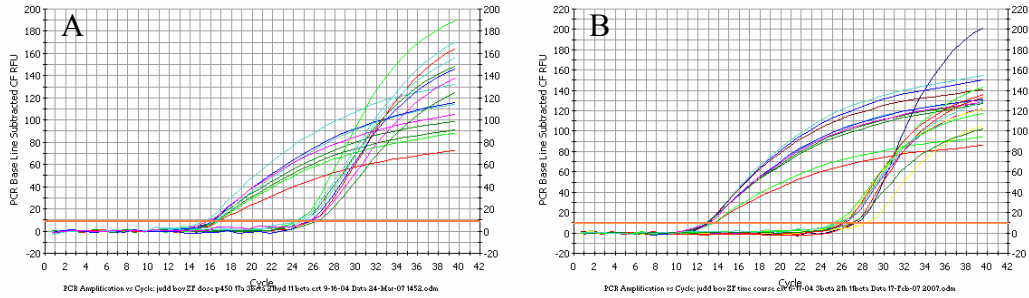


Fig. 23: Representation of the light emitted by SYBR green during the real time PCR for 11 β hydroxylase ZF dose dependent (A) and time dependent (B) reactions.

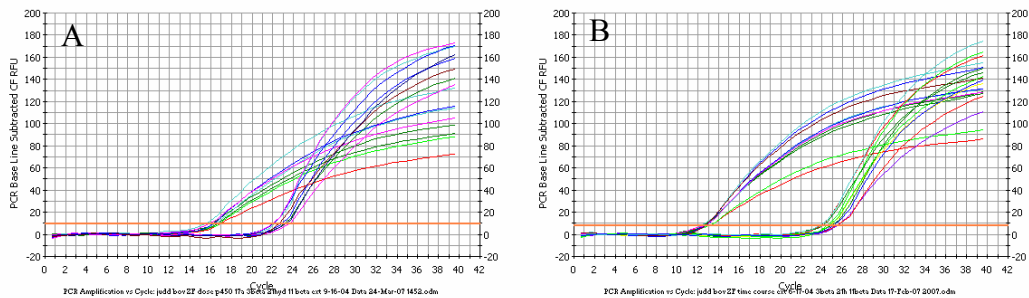


Fig. 24: Representation of the light emitted by SYBR green during the real time PCR for 21 hydroxylase ZF dose dependent (A) and time dependent (B) reactions.

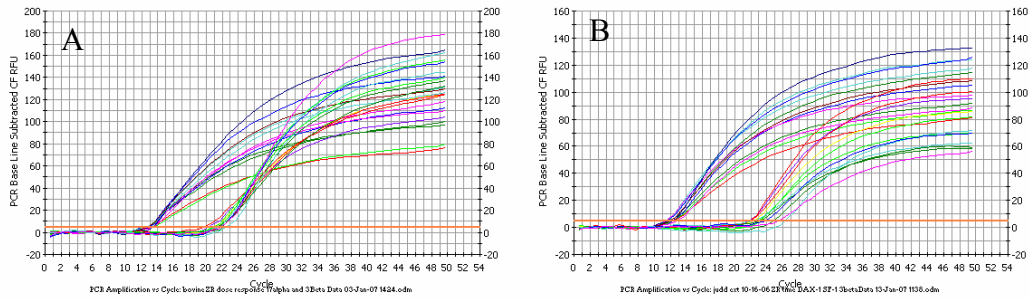


Fig. 25: Representation of the light emitted by SYBR green during the real time PCR for 3 β HSD2 ZR dose dependent (A) and time dependent (B) reactions.

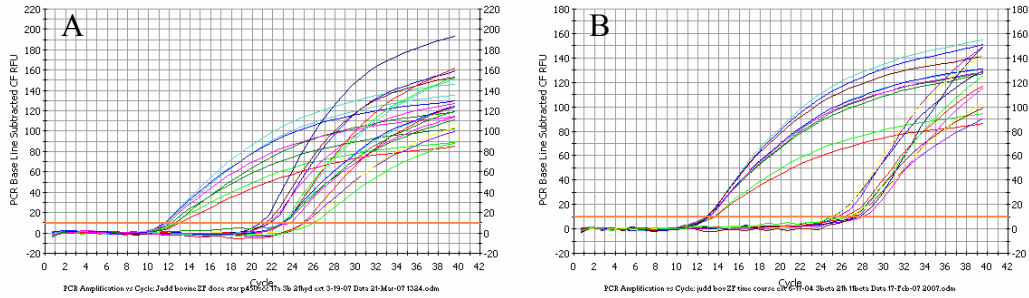


Fig. 26: Representation of the light emitted by SYBR green during the real time PCR for 3 β HSD2 ZF dose dependent (A) and time dependent (B) reactions.

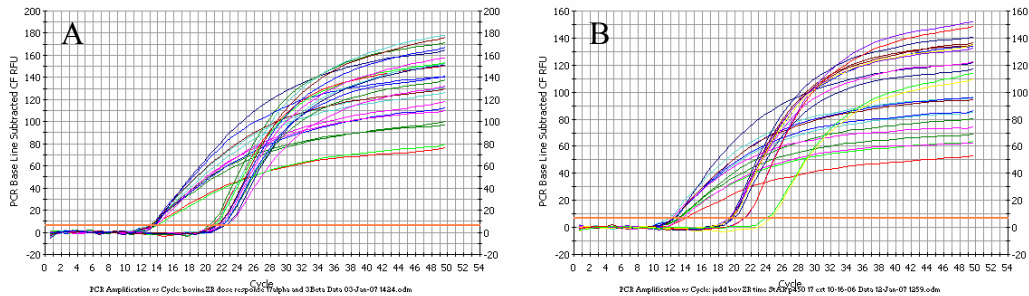


Fig. 27: Representation of the light emitted by SYBR green during the real time PCR for 17 α hydroxylase bovine ZR dose dependent (A) and time dependent (B) reactions.

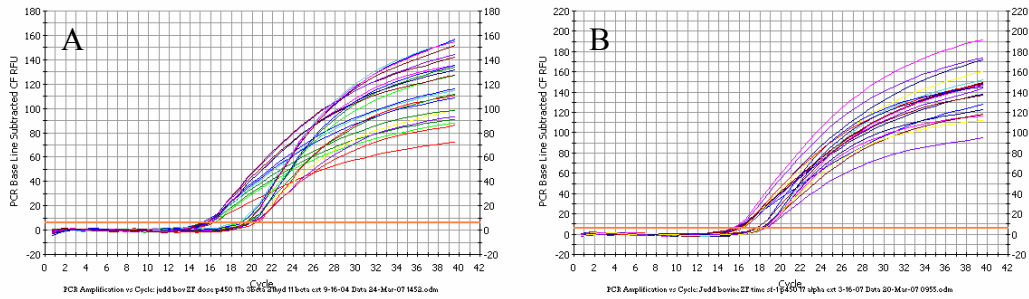


Fig. 28: Representation of the light emitted by SYBR green during the real time PCR for 17 α hydroxylase bovine ZF dose dependent (A) and time dependent (B) reactions.

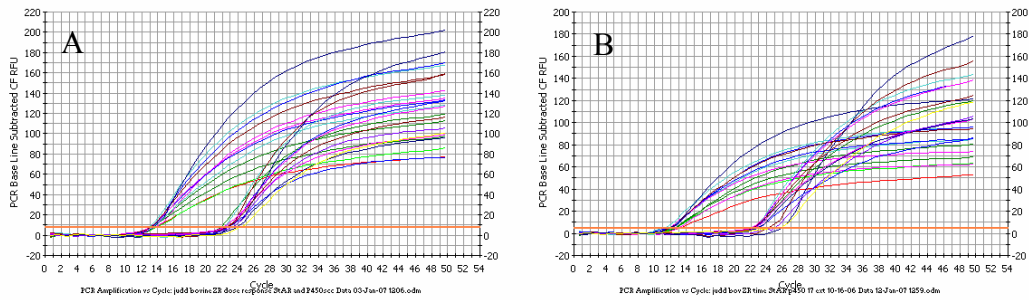


Fig. 29: Representation of the light emitted by SYBR green during the real time PCR for P450sc bovine ZR dose dependent (A) and time dependent (B) reactions.

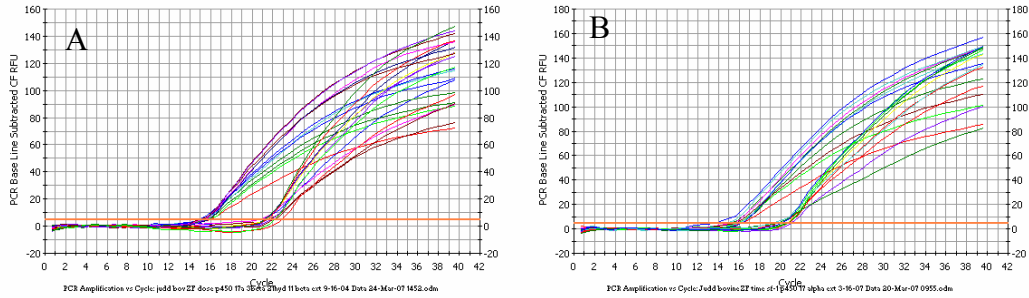


Fig. 30: Representation of the light emitted by SYBR green during the real time PCR for P450scc bovine ZF dose dependent (A) and time dependent (B) reactions.

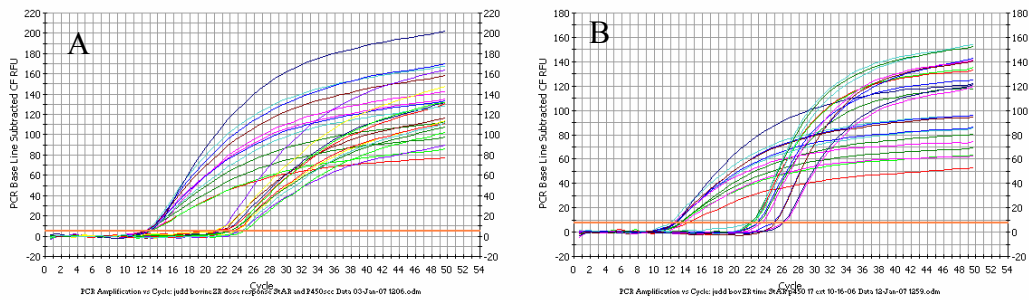


Fig. 31: Representation of the light emitted by SYBR green during the real time PCR for StAR bovine ZR dose dependent (A) and time dependent (B) reactions.

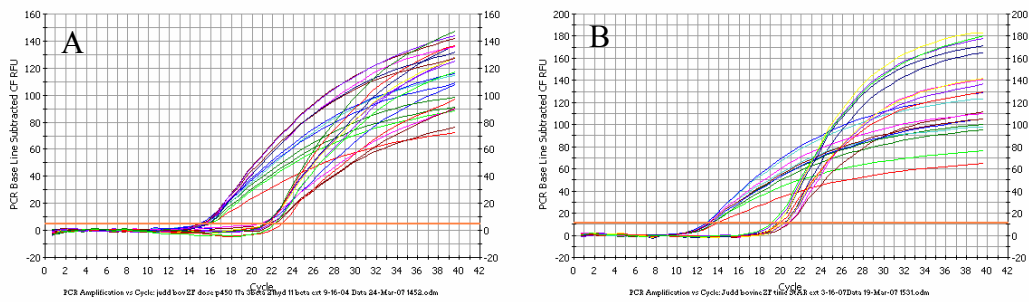


Fig. 32: Representation of the light emitted by SYBR green during the real time PCR for StAR bovine ZF dose dependent (A) and time dependent (B) reactions.

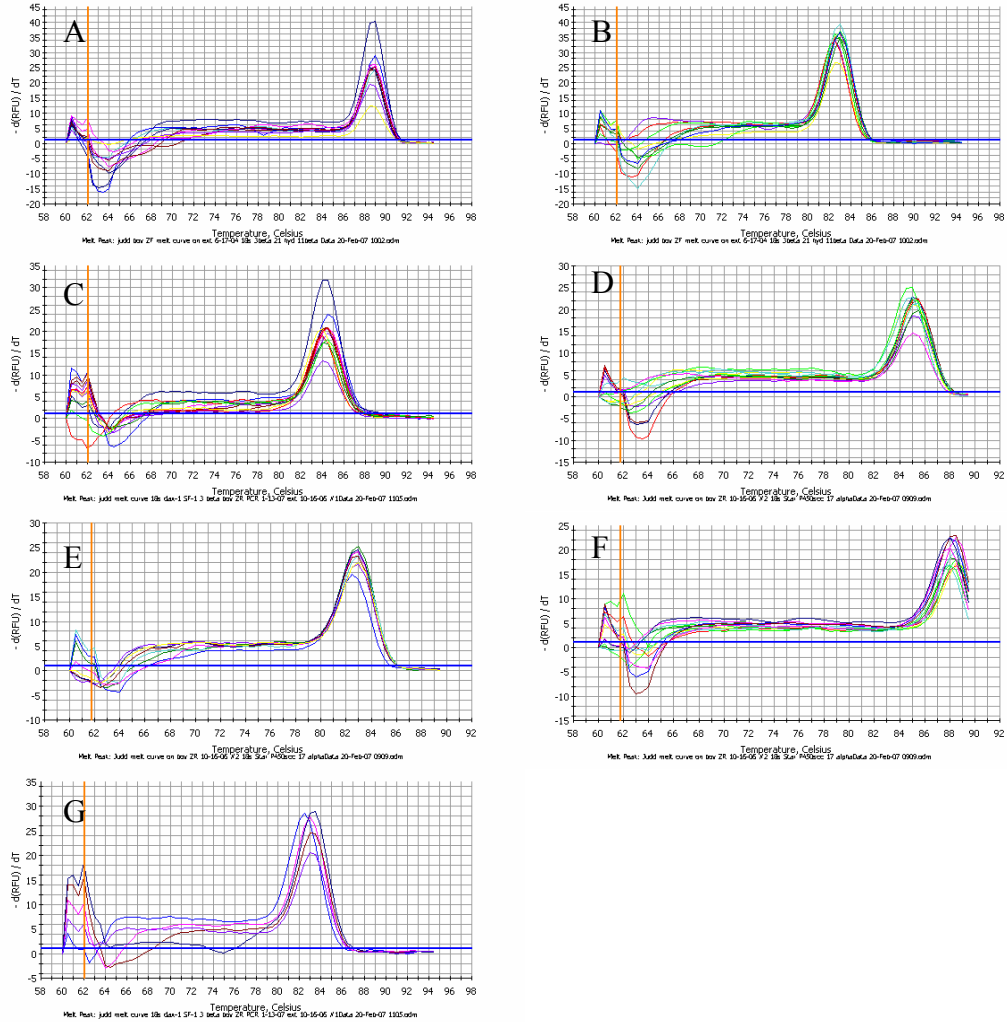


Fig. 33: Melt Curves of the Real Time PCR Products
 Fig. 31A: 11 β hydroxylase bovine melt curve. Fig. 31B: 21 hydroxylase bovine melt curve. Fig. 31C 3 β HSD2 bovine melt curve Fig. 31D: 17 α hydroxylase bovine melt curve Fig. 31E: P450scc bovine melt curve Fig. 31F: StAR bovine melt curve Fig. 31G: 18s bovine melt curve

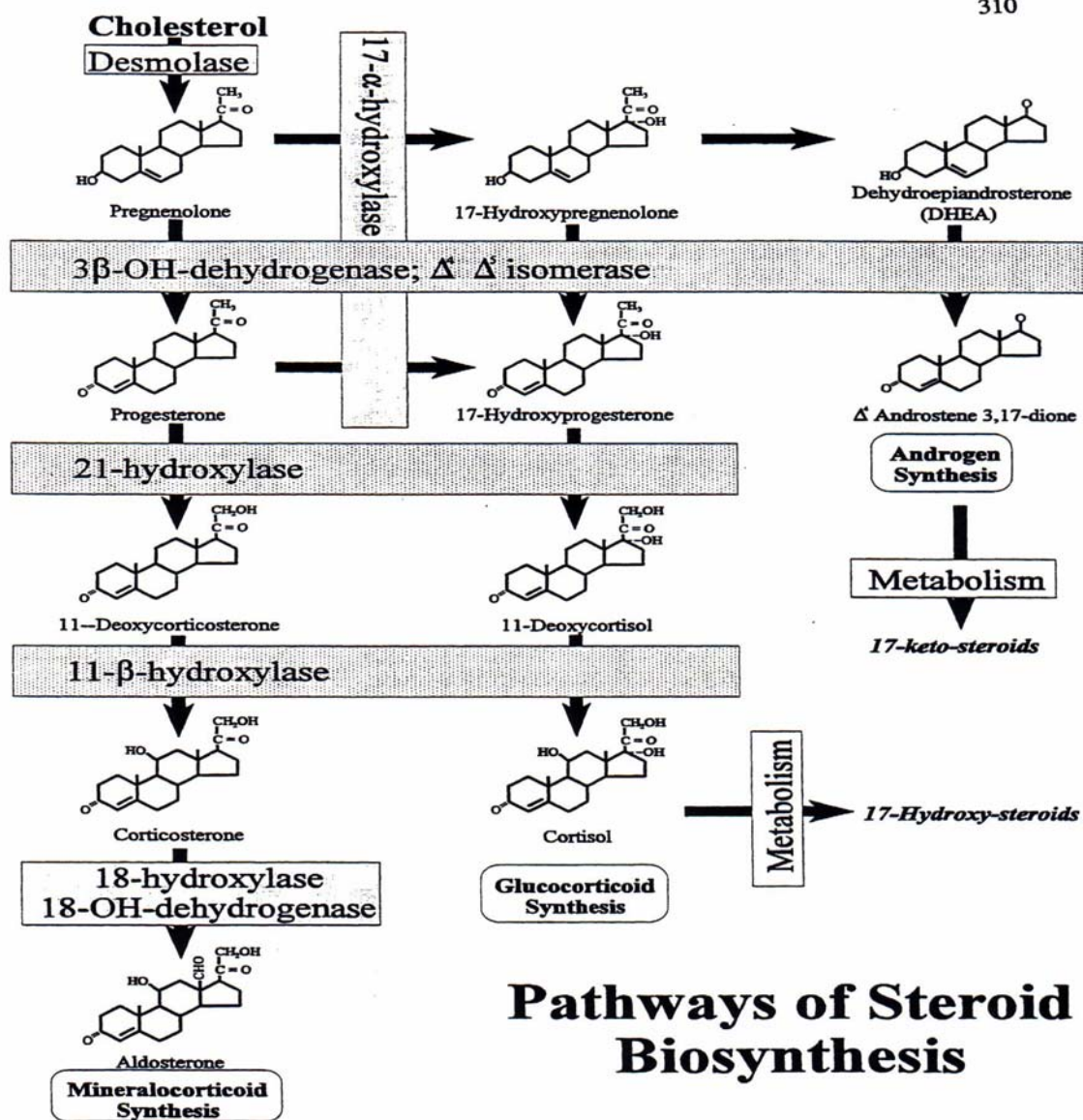


Diagram 1: Steroidogenic Pathway

Diagram of adrenal steroidogenic pathways and the enzymes involved (18).

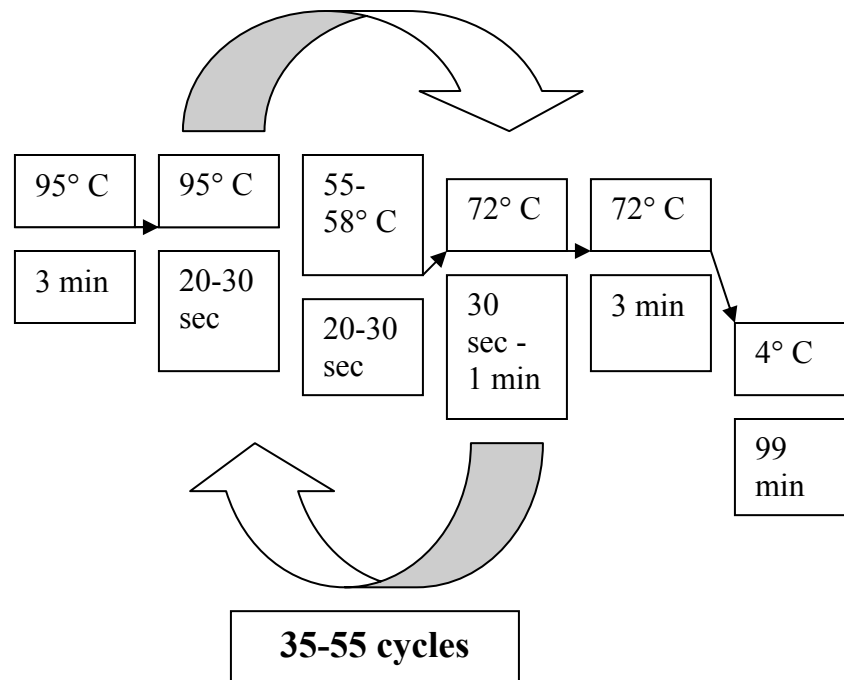


Diagram 2: PCR Temperatures and Times

Diagram of temperatures and cycles used for standard and real time PCR thermocyclers. Different times and cycles were used for standard PCR than real time PCR. Lower temperatures and times were used for real time PCR. Each primer had an optimal annealing temperature.

Gene	Sequence	Strand
Human P450scc	GAGACCCATAGGAGTCCTGTTGAA	Anti-sense
	AACCAGGAGGTGATGGCTCC	Sense

Table 1: Human primers used for real time PCR

Gene	Sequence	Strand
Bovine StAR	TGGCATGGCCCACTCTATG	Sense
	CCAGGTGAGTTTGGTCCTTGA	Anti-sense
Bovine P450scc	TCTCTTCAAGTTCGAGGGATCCT	Sense
	CTCCAATGGGTTTCTGATAATATCG	Anti-sense
Bovine 17 α Hydroxylase	TGTGGCCCCCTACGCTGAT	Sense

	CAACGTCTGTGCCTTTGTCAAT	Anti-sense
Bovine 3 β HSD	ACCAATCACTGCAAGTTCTCAAGA	Sense
	GGCCCTCAAGGCCAGAAT	Anti-sense
Bovine 21 Hydroxylase	TGCCTTTCACGACTGTGTTCA	Sense
	AAGAACCTGAGAAAAGGACCAATGT	Anti-sense
Bovine 11 β Hydroxylase	GCGGCTTGGCCTTTTCA	Sense
	CAAAGTGCTCCCTCCACATGT	Anti-sense

Table 2: Bovine primers used for real time PCR

Gene	Sequence	Strand
Human DAX-1	AGGGGACCGTGCTCTTTAAC	Sense
	ATGATGGGCCTGAAGAACAG	Anti-sense
Human SF-1	GCAGAAGAAGGCACAGATTC	Sense
	TGGGTA CT CAGACTTGATGG	Anti-sense
Human StAR	CCAGATGTGGGCAAGGTG	Sense
	CCTCTGCGCTTGGTACAGC	Anti-sense
Human P450 _{scc}	CCTGCAGTGGCACTTGTATG	Sense
	GGTCATCTCTAGCTCAGCGA	Anti-sense
Human 17 α Hydroxylase	CATGCTGGACACACTGATGC	Sense
	CCTCCAGGCCTGGCGCACCT	Anti-sense
Human 3 β HSD	TGGAGCTGCCTTGTGACAGGA	Sense
	CATCTGGAATCAAGGCGGAG	Anti-sense
Human 21 hydroxylase	TGCCTGCCTATTACAAATGT	Sense
	GGTGAAGCAAAAAAACCACG	Anti-sense
Human 11 β Hydroxylase	TGCGCGTGTTCCTCTACTCT	Sense
	AGAGACGTGATTAGTTGATG	Anti-sense

Table 3: Human primers used for standard PCR

Gene	Sequence	Strand
Bovine DAX-1	AGGGGACCGTGCTCTTTAAC	Sense
	ATGATGGGCCTGAAGAACAG	Anti-sense
Bovine SF-1	GCAGAAGAAGGCACAGATTC	Sense
	TGGGTACTCAGACTTGATGG	Anti-sense
Bovine StAR	CCTCTCTACAGCGACCAA	Sense
	TCGTGAGTGATGACCGTG	Anti-sense
Bovine P450 _{scc}	AACGTCCCTCCAGAACTGTACC	Sense
	CTTGCTTATGTCTCCCTCTGCC	Anti-sense
Bovine 17 α Hydroxylase	TACTGATCCAAGCCAAGGTG	Sense
	GCGCACCTCGATCTTCAC	Anti-sense
Bovine 3 β HSD	GGCCGTCTTGGATGATCT	Sense
		Anti-sense
Bovine 21 Hydroxylase	CGTGACCATCCAGAAGGAAT	Sense
	GGTGAAGTAGGAACGCCACA	Anti-sense
Bovine 11 β Hydroxylase	ACGCTGGTGAAGGTCCTACT	Sense
	TCAGGGCTCGTAGGAGAAAA	Anti-sense

Table 4: Bovine primers used for standard PCR

References

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Stephen Mcilmoil

2033 N. 650 W.
Provo UT 84604
(801) 369-6900
sm300wby@hotmail.com

ACADEMIC PREPARATION:

M.S. in Physiology and Developmental Biology - College of Biology and Agricultural Studies, Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT 2007

Concentrations: Human Physiology and Endocrinology

Thesis: Regulation of Adrenal Steroidogenesis by interleukin-6

Advisor: Dr. Allan M. Judd

B.S. in Biology, Brigham Young University, Provo, UT 2005

Concentrations: Physiology

LANGUAGES:

- Czech

PROFESSIONAL EXPERIENCE:

Teaching Assistant

Department of Physiology and Developmental Biology, Provo UT
Courses: PDBio 363: Advanced Physiology Laboratory

Research Assistant

Department of Physiology and Developmental Biology, Provo UT
Duties: Standard and Real time PCR, Western Blot

POSTERS PRESENTED AT CONFERENCES:

Induction of mRNA Expression of the Adrenal Steroidogenic Enzymes by Interleukin-6. Presented at the Annual Endocrine Society Conference, Boston, MA, 2006

Interleukin-6 Inhibition of Bovine Adrenal Androgen Release Involves Suppression of Steroidogenic Enzymes and SF-1 Expression and Augmentation of DAX-1

Expression. Presented at the Annual Endocrine Society Conference, Toronto, Canada
2007

Future Endeavors:

I will be attending Western University of Health Sciences College of
Osteopathic medicine of the Pacific, Fall 2007 – 2011. I planning to
specialize in internal medicine and practice in Oregon.

REFERENCES:

Allan M. Judd, Advisor/Professor
Department of Physiology
Brigham Young University, Provo UT
Allan_judd@byu.edu