Investigation of the Role of DAX-1 in the HPA Axis in Human Adrenal Cells

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This Biology Honors Thesis

Investigation of the Role of DAX-1 in the

HPA Axis in Human Adrenal Cells

by

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is submitted in partial fulfillment of the requirements for the

Biology Honors Program

at the

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Committee member
ABSTRACT

DAX-1 is a nuclear hormone orphan receptor that plays a key role in the development of reproductive tissues and steroid hormone production. The hypothalamic-pituitary-adrenal axis is the pathway for steroid hormone production, primarily glucocorticoids, mineralocorticoids, and adrenal androgens, in the human body. The mechanisms of DAX-1 in hormone production in the adrenal cortex of the HPA axis are not completely understood and, therefore, were the focus of this honors thesis research project. Due to the high level of DAX-1 expression, SW13 adrenal carcinoma cells were conducted for these experiments. We investigated whether glucocorticoids, specifically the synthetic glucocorticoid dexamethasone, had any positive or negative feedback on DAX-1 expression in SW13 cells. To identify key proteins that regulate DAX-1 gene expression following dexamethasone exposure, chromatin immunoprecipitation (ChIP) assays were utilized. My results, specifically utilizing quantitative PCR, found that dexamethasone-treated SW13 cells increased DAX-1 expression, with the highest level of upregulation following treatment with $10^{-6}$M dexamethasone. A time course treatment of the SW13 cells found that the maximum change in DAX-1 expression required exposure to dexamethasone ($10^{-6}$M) for 6 hours. The ChIP results show expressions of those proteins playing a role in the DAX-1 promoter, but the results were inconclusive. These include the EWS/FLI1 oncoprotein (Mendiola et al., 2006), beta-catenin (Mizusaki et al., 2003), Androgen Receptor (Lanzino et al., 2013), as well as the orphan NHR, SF1 (Kawabe et al., 1999). The results presented in this thesis demonstrate the effect of glucocorticoid exposure on DAX-1 expression, providing a more complete picture of the H-P-A axis and the role of DAX-1 in this pathway. Understanding the role of DAX-1 in this important physiological process and its connection to diseases such as Adrenal Hypoplasia Congenita (AHC) may provide an alternative therapeutic target in the future.
INTRODUCTION

Overview of DAX-1

Nuclear hormone receptors (NHRs) are transcription factors that are regulated by hormone binding. NHRs include three different subgroups, steroid receptors, non-steroid receptors, and orphan receptors. These proteins play critical roles in various physiological processes such as stem cell differentiation, sex determination and development, and metabolism and homeostasis. In general, NHRs function by binding their cognate ligand, typically a hormone that easily diffuses through the plasma membrane, and, upon ligand binding, forming a ligand-NHR dimer complex. The NHR dimer enters the nucleus and binds to its specific target gene sequence within the promoter region of the DNA (Figure 1). This results in either upregulation or downregulation of specific target genes and, ultimately, an overall change in cellular response.

The main NHR studied in the Tzagarakis-Foster lab is DAX-1, which stands for Dosage-Sensitive Sex reversal (DSS), Adrenal Hypoplasia Congenita (AHC), a critical region on the X chromosome, gene 1 (Mendiola 2006). DAX-1 is an orphan nuclear receptor encoded by the NR0B1 gene and is involved in sex determination and steroid hormone synthesis in humans. The DAX-1 gene has been shown to play a key role in transcription and typically functions as a negative regulator of the transcription of various hormone-responsive genes (Lanzino 2013).

Figure 1: A depiction of nuclear hormone receptors signaling. There are multiple steps from the ligand nuclear hormone receptor binding resulting in a cellular response (Dishington, 2017).

Overview of the HPA axis?
The hypothalamic-pituitary-adrenal axis (HPA) is the main pathway of steroid hormone production in humans. It consists of the hypothalamus (H), anterior pituitary (P), and adrenal cortex (A). One of the main hormones produced in this pathway is the glucocorticoid, cortisol (Figure 2). Cortisol is a steroid hormone that regulates a human’s ability to control stress, regulate glucose metabolism, and suppress immune responses. Glucocorticoids are essential to life and play a key role in functions of the body like appetite, blood pressure, and sleep (Gummow 2006). Another class of hormones produced via the HPA pathway is the mineralocorticoids, with aldosterone being the primary hormone in this class. Aldosterone is known to be important in balancing salt concentration in the body. The HPA axis is positively regulated through the release of corticotropin-releasing factor (CRF) from the hypothalamus and adrenocorticotropic hormone (ACTH) that is released from the anterior pituitary (Ikeda 2001). The HPA axis is regulated through negative feedback through the secreted steroid hormones (such as glucocorticoids and mineralocorticoids) that inhibit the secretion of CRF or ACTH, thereby shutting off this pathway (Guo 1995).

Figure 2: The general pathway of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis includes the hypothalamus, anterior pituitary, and adrenal cortex. The axis is regulated through both positive and negative feedback by cortisol. (Vetter 2011).
Mutations of DAX-1 can be linked to disruptions within the HPA axis and can result in various diseases. Adrenal hypoplasia congenita (AHC) is an X-linked disorder that results from mutations in the DAX-1 gene. AHC can result in failure to develop the adrenal cortex, which can be fatal, and a perturbation in puberty later in human life. AHC is a rare disease and is commonly misdiagnosed. Due to this misdiagnosis, there are various delays in treatment that could be considered life-threatening (Reutens 1999). AHC is known to have disruptions with development disorders of hypothalamic GnRH cells, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) cells (Ouyang 2021).

Honors Thesis Research Project

This Thesis research project focused on the expression of the DAX-1 gene in the human adrenal cortex. This gland is important in the signaling and production of steroid hormones. The original proposal for my thesis project was to focus on the role of DAX-1 within the hypothalamus by using a neuroblastoma cell line (SK-N-BE(2)), however, this cell line was not available at the time this research was initiated. As an alternative, I carried out my thesis research using the human adrenal carcinoma cell line, SW13. For these experiments, SW13 cells were exposed to the glucocorticoid hormone, dexamethasone, in order to determine if exposure to this steroid hormone resulted in a change in DAX-1 expression. To further investigate the factors that play critical roles in the regulation of DAX-1 expression following glucocorticoid exposure, chromatin immunoprecipitation (ChIP) assays were employed. Given the impairment in steroid hormone production in patients with AHC, examination of the role of glucocorticoids, the primary hormone produced in the human adrenal cortex, on DAX-1 expression in expected to provide a better understanding of DAX-1 in the HPA axis.

METHODS

Cell culture and dexamethasone treatment

SW13 adrenal carcinoma cells were originally obtained from American Type Culture Collection (ATCC). Cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum and 1X penicillin/streptomycin (Fisher Scientific) and grown at 37°C in a humidity-controlled cell culture incubator with 5% CO₂. Cells were checked regularly for confluency.

Dexamethasone dose-response and time course
SW13 cells were seeded at 1 x 10^4 cells/ml into a six-well plate with varying concentrations of dexamethasone. The experiment was set up as follows: day 0 was the day of cell seeding, day 1 was the day the complete media was switched to serum-free media to allow the cells to synchronize by entering G_0, day 2 was an exposure of the SW13 cells to dexamethasone and day 3 was the day of cell harvesting for RNA isolation. For the dexamethasone dose-response experiments, increasing concentrations of dexamethasone were added to each well of cells starting with 10^{-9} M at the lowest concentration and increasing to 10^{-5} M at the highest concentration. One well was treated with 0.01% ethanol as the vehicle control.

For the dexamethasone time course, cells were plated as described and treated with 10-6M dexamethasone for 0 minutes, 30 minutes, 60 minutes, 6 hours, 12 hours, and 24 hours. Following treatment, cells were harvested for RNA isolation.

**RNA isolation**

Following dexamethasone treatment, SW13 cells were harvested for total RNA isolation. The Monarch Total RNA miniprep kit (New England Biolabs, Inc) was used according to the manufacturer’s instructions. SW13 cells were lysed using 600 μl of RNA lysis buffer and a syringe to break up the chromatin. Genomic DNA is removed using the gDNA removal column and flowthrough is collected and transferred to an RNA isolation column. Following centrifugation, the column was washed several times to remove any non-RNA material, and the column was treated with a DNaseI to eliminate any remaining genomic DNA. Another round of column washes was performed, columns were transferred to new RNase-free microcentrifuge tubes and RNA was eluted with nuclease-free water. RNA concentration was determined by spectrophotometry on the NanoDrop spectrophotometer (Fisher Scientific).

**cDNA synthesis**

Following RNA isolation, cDNA was synthesized using the QuantaBio cDNA Supermix following the manufacturer’s instructions. Briefly, a total of 1 μg total RNA was used as input for each cDNA synthesis reaction along with 4 μL qScript cDNA SuperMix (5X). Samples were incubated as follows in a thermal cycler: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and held at 4°C. Samples were stored at -20°C until PCR analysis.

**End-point PCR**
Samples were prepared using GoTaq 2x Master Mix (Promega), and primers were designed to detect the DAX-1 gene and GAPDH as a control. PCR conditions and primers (see Table 1 and Table 2 below) were obtained from Integrated DNA Technologies. Following PCR, products were analyzed by agarose gel electrophoresis using a 2% agarose gel made in 1X TAE plus ethidium bromide. Products were visualized using the GelDoc system (BioRad) and ImageQuant software (BioRad).

### Table 1: PCR primers

<table>
<thead>
<tr>
<th>PCR Primer Set:</th>
<th>Forward Primer sequence</th>
<th>Reverse Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers to detect DAX-1 gene expression</td>
<td>5’ - GAC TCC AGT GGG</td>
<td>5’ - ATG ATG GGC CTG</td>
</tr>
<tr>
<td></td>
<td>GAA CTC AG - 3’</td>
<td>AAG AAC AG - 3’</td>
</tr>
<tr>
<td>Primers to detect GAPDH gene expression (positive control)</td>
<td>5’ - CCA TCA CCA TCT</td>
<td>5’ - AGA GAT GAT GAC</td>
</tr>
<tr>
<td></td>
<td>TCC AGG AGC G - 3’</td>
<td>CCT TTT GGC - 3’</td>
</tr>
<tr>
<td>Cut primers used for ChIP</td>
<td>5’ - AAT GCA GGA ACA</td>
<td>5’ - GGC AGC GAG CAG</td>
</tr>
<tr>
<td></td>
<td>GAA GAA AAC CAA ATA -</td>
<td>GAT GTA AAA GTG - 3’</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td></td>
</tr>
<tr>
<td>Long primers used for ChIP</td>
<td>5’ - GAG GAT GGG AGG GAG GAG GGA AAA AGT - 3’</td>
<td>5’ - AGG GCA GGG GAA AAG AGG AAA CAT - 3’</td>
</tr>
</tbody>
</table>

### Table 2: PCR conditions

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>End-point PCR</strong></td>
<td>95°C x 3 minutes 1 repeat</td>
</tr>
<tr>
<td></td>
<td>95°C x 30 seconds</td>
</tr>
<tr>
<td></td>
<td>55°C x 30 seconds</td>
</tr>
</tbody>
</table>
Temperature Gradient

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 °C x 1 minute</td>
<td></td>
</tr>
<tr>
<td>72 °C x 5 minutes 1 repeat</td>
<td></td>
</tr>
<tr>
<td>4 °C infinite hold</td>
<td></td>
</tr>
</tbody>
</table>

Quantitative PCR

Quantitative PCR, or qPCR, reactions were prepared using SsoAdvanced SYBR Green Master Mix from BioRad. PCR Primer and qPCR parameters are shown above in Table 1 and Table 3 below.

Table 3: qPCR conditions

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR</td>
<td>98 °C x 30 seconds 1 repeat</td>
</tr>
<tr>
<td></td>
<td>98 °C x 10 seconds 40 repeats</td>
</tr>
<tr>
<td></td>
<td>60 °C x 30 seconds</td>
</tr>
</tbody>
</table>

To analyze the qPCR data, the ΔCT method was utilized. The average DAX-1 Ct
values were subtracted from the average of GAPDH. Finally, the ΔΔCT was calculated, which is derived by the following equation = $2^{\Delta \Delta Ct}$.

*Chromatin Immunoprecipitation (ChIP) Assays*

In order to analyze which proteins are bound to the DAX-1 promoter in the SW13 cells following dexamethasone treatment, ChIP assays were performed using the Magna ChIP kit (Millipore). SW13 cells were grown to 70-80% confluency on two 15 cm dishes. Following dexamethasone treatment, cells were harvested for ChIP analysis according to the manufacturer’s instructions. After 24 hours of incubation, cells were treated with 1% formaldehyde (Fisher Scientific). Cell lysates were sonicated on ice, 20 times for 10 seconds with a 20-second pause. Immunoprecipitations were performed by incubating fragmented chromatin with appropriate antibodies overnight at 4°C. ChIP reactions contained the following antibodies: (WHAT ANTIBODIES WERE USED and from what company?) An aliquot of the cell lysates was used to isolate the total input DNA. Amplifications of the immunoprecipitated DNA were performed using end-point PCR.

**RESULTS**

*Aim 1: Investigate the hormonal controls that regulate the expression of DAX-1 in the human adrenal gland*

This aim has experiments tailored to see the effects of glucocorticoids on DAX-1 expression. A dosage response using dexamethasone was done to determine the controls of DAX-1 on adrenal carcinoma cells. This experiment was done three times in order to seek the validity of the data.
Figure 3: This was the first run of qPCR with different concentrations of treatment of glucocorticoids on SW13 adrenal cells.

The figure showcases an upward trend with the increasing strength of the concentration of glucocorticoids (Figure 3). There seems to be a threshold of $10^6$ for how much glucocorticoids can affect the expression of $DAX-1$.

Figure 4: This was the second run of qPCR with different concentrations of treatment of glucocorticoids on SW13 adrenal cells.
This also shows an upward trend with the increasing strength of the concentration of glucocorticoids. There seems to be the same threshold of $10^6$ how much glucocorticoids can affect the expression of $DAX-1$.

Figure 5: This was the third run of qPCR with different concentrations of treatment of glucocorticoids on SW13 adrenal cells.

There is an upward trend during this run that has the increasing strength of the concentration of glucocorticoids. There seems to be a difference in the results compared to my first two PCRs, but the upward trend is still apparent.

Another experiment that was done was a time course. A time course allows for the determination of the most $DAX-1$ expression in the human adrenal cells. This determined that six hours was the ideal time to treat the cells with glucocorticoids.
Figure 6: This was the run of qPCR with different time treatments of glucocorticoids of $10^{-6}$ concentration on SW13 adrenal cells.

There seems to be a peak of expression at 6 hours. After this, there seems to be a giant drop in \textit{DAX-1} expression after 6 hours.

\textit{Aim 2: Identify factors in the transcription complex that mediate \textit{DAX-1} expression impacting the HPA axis.}
Figure 7: Here are the two primers found to use in the ChIP protocol.

The first primer is the hDAX-1 promoter, which has a larger sequence length. The second primer is the DAX-1 ARE promoter which is a smaller sequence within the DAX-1 promoter primer sequence.
Figure 8: This is the PCR of the optimization of the hDAX-1 promoter sequence.

The temperature gradient was from 60°C to 50°C. The lane that had the clearest band was the first lane. The annealing temperature for the hDAX-1 promoter was 60°C. This would be the best temperature to use during the last step of the ChIP protocol.

Figure 9: This is a PCR of the optimization of the DAX-1 ARE promoter sequence.

The temperature gradient was from 60°C to 50°C. The lane that had the clearest band was the third to last lane. The annealing temperature for the DAX-1 ARE promoter was 52.3°C. 52.3°C would be the best temperature to use in the PCR step of the ChIP protocol.

**DISCUSSION**

The overall results for the first aim of the experiment showcase how to identify different controls that are used to affect DAX-1 expression. The concentration that stimulates the highest amount of DAX-1 expression with the treatment of dexamethasone is 10^-6. The ideal time for treatment infection is 6 hours, which resulted in the highest amount of DAX-1 expression. These results help with a better understanding of the regulation of the DAX-1 promoter and can use this information for future experiments.

Temperature gradients were used in order to determine the best annealing temperature for primers. By optimizing the two primers, hDAX-1 and DAX-1 ARE, the best annealing
temperatures were found. On the hDAX-1 promoter sequence, the best temperature was 60°C. In contrast, the best temperature for the DAX-1 ARE was 52.3°C. The optimization of these primers is essential to have a clear polymerase chain reaction (PCR). This will aid in future experiments like the end-point PCR step of ChIP. The ChIP protocol showcased what protein-DNA interactions there were.

A full protocol of ChIP was completed but there was no viable data. Primer dimers were the only result on the gel. This was identified because the bands were smaller than the ladder on the gel. These primer dimers are the results of nonspecific elongated primers. Due to this result, another ChIP protocol has to be done. By working with one of the graduate students, Lana, in the research lab, back-to-back ChIP protocols were run to identify the issues of the protocol. Sonification of the chromatin was done in order to ensure the shearing of the enzymes but this run was unable to get any viable data. The protocol will be run again with the sonification of the chromatin plus the enzymatic shearing solution and possibly a higher amount of antibody during the immunoprecipitation step. Western blot could be used for further protein analysis.

More knowledge about how to control DAX-1 in the adrenal carcinoma cell line (SW13) was found. The results showcase the hormonal controls that affect DAX-1 using glucocorticoids and try the better understand the mechanism behind DAX-1. This data showcases how DAX-1 fits within the HPA axis in humans. This could be the first step in trying to control DAX-1 in patients with adrenal hypoplasia congenita. Glucocorticoids can be used With the work of Lana and me, we have introduced a new branch of research within the Transcriptional Regulation of Eukaryotes Lab.

Some future directions that this experiment may take are further experiments on how DAX-1 affects different parts of the HPA axis. Like my original idea for my thesis, there could be more research done on how DAX-1 affects the hypothalamus because it is the first part of the HPA axis. By grasping a better understanding of how the DAX-1 mechanism works, this orphan nuclear hormone receptor could be used to regulate the production of hormones and could be used for future treatment options for various cancers and other diseases.

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REFERENCES


