ANDROGEN REGULATION OF INSULIN SIGNALING IN 3T3-L1 INDUCED ADIPOCYTES

An Undergraduate Research Scholars Thesis

by

BRIANA BRYSON

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TABLE OF CONTENTS

Page

ABSTRACT.. 1

ACKNOWLEDGEMENTS 3

NOMENCLATURE 4

SECTION

I INTRODUCTION 6

II METHODS 9

Cell culture 9

RNA extraction and quantitation, cDNA synthesis, protein quantitation, and real time PCR 10

Glucose determination in media 11

Statistical analysis 11

III RESULTS 12

Real time PCR analysis 12

Glucose concentration in cell culture media 12

IV CONCLUSION 15

REFERENCES 16

ABSTRACT

Androgen Regulation of Insulin Signaling in 3T3-L1 Induced Adipocytes

Briana Bryson
Department of Animal Science
Texas A & M University

Research Advisor: Dr. Annie Newell-Fugate
Department of Veterinary Physiology and Pharmacology

Polycystic ovary syndrome (PCOS), the most common endocrine disorder of reproductive age women, affects obese women disproportionately.[1] This syndrome is characterized by hyperandrogenemia, cystic ovaries, and/or oligo/anovulation.[2] Hyperandrogenic conditions such as PCOS are often associated with insulin resistance and hyperinsulinemia, particularly in obese women.[3] However, it is unknown whether excess androgens play a direct role in the promotion of insulin resistance in obese females. We hypothesize androgens, independent from obesity, cause disruption of insulin signaling in adipocytes at the level of the androgen receptor (AR). Using the murine 3T3-L1 pre-adipocyte cell line, we assessed the effects of dihydrotestosterone (DHT; 10^{-6} M), a potent, non-aromatizable androgen, on insulin signaling in induced adipocytes. Identification of the role androgens may play in adipocyte insulin signaling will help elucidate the relationship between hyperandrogenemia and hyperinsulinemia and may provide a foundation for the development of new pharmaceuticals designed to target both hyperinsulinemia and hyperandrogenemia in obese female patients. We assessed mRNA transcript expression of forkhead box A1 (FOXA1), forkhead box O1 (FOXO1), insulin receptor (INSR), and protein kinase B isoform 2 (Akt2) relative to glyceraldehyde phosphate dehydrogenase (GAPDH) in cultured adipocytes and glucose

present in cell culture media to determine how DHT may affect the insulin signaling in 3T3-L1 induced adipocytes. Our data revealed no significant difference in mRNA transcript expression of FOXO1, INSR, and Akt2 relative to GAPDH in control and DHT-treated 3T3-L1 induced adipocytes, and we found that FOXA1 could not be detected. Additionally, there was no significant difference in media glucose concentrations collected from wells containing DHT-treated and control 3T3-L1 induced adipocytes. While it is possible DHT does not alter insulin signaling in 3T3-L1 induced adipocytes, it recommended that additional replications of this experiment be completed in order to increase the statistical power.

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NOMENCLATURE

Akt	Protein Kinase B
Akt2	Protein Kinase B Isoform 2
ANOVA	Analysis of Variance
AR	Androgen Receptor
ATCC	American Type Culture Collection
DEPC	Diethylpyrocarbonate
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
foxa1	Forkhead Box A1
foxo2	Forkhead Box O2
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GLUT4	Glucose Transporter 4
HRP	Horseradish Peroxidase Concentrate
IBMX	3-isobutyl-1-methylxanthine
INSR	Insulin Receptor
IRS1	Insulin Receptor Substrate 1
IRS2	Insulin Receptor Substrate 2
LSM	Least Squares Mean
PCOS	Polycystic Ovary Syndrome

PKB	Protein Kinase B
PKCε	Protein Kinase C Epsilon Type
PPAP2A	Phosphatidic Acid Phosphatase 2a
qPCR	Quantitative Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
TC	Tissue Culture

SECTION I

INTRODUCTION

Up to 70 % of Americans are overweight or obese, and the age-adjusted rate of obesity in women is nearly 35 %.[4] In women, increased visceral fat deposition, insulin resistance, glucose intolerance, and type II diabetes are all closely associated with polycystic ovary syndrome.[3] PCOS, characterized by hyperandrogenism, chronic oligo/anovulation and/or ovarian cysts, affects about 1 in 10 women of reproductive age.[1] Obese women represent 50 to 70 percent of PCOS cases[5], and tend to express more aggravated symptoms of the disorder.[1] Hyperinsulinemia, though not considered a defining symptom of PCOS, is also closely associated with the disorder and more pronounced in obese patients.[6] Whether excess androgens play a role in the promotion of insulin resistance in obese females is unknown. Available treatment options for PCOS are inefficient in that they target the various symptoms rather than the syndrome itself.[6] Determining the effect of androgens on insulin signaling in obese females is critical in order to facilitate development of novel pharmaceuticals to simultaneously treat both insulin resistance and hyperandrogenism in obese females.

Most of the research performed thus far on the role of androgens in insulin signaling pathways has focused on men, particularly in the area of prostate cancer. A study using prostate cells showed that stimulation of the androgen receptor (AR) via removal of the AR repressor protein, forkhead box O1 (foxo1), from the nucleus leads to increased insulin signaling.[5] However, the role of androgens in glucose homeostasis is sexually dimorphic, with insulin resistance occurring in females with elevated serum androgen levels but in males with

decreased serum androgen levels.[3] A single study in lean women found that in-vitro treatment of differentiated primary pre-adipocytes with testosterone caused insulin resistance via insulin-stimulated phosphorylation of protein kinase C ζ, which glucose into the cell via the glucose transporter type 4 (GLUT4) transporter.[9] The pathway by which androgens may mediate this insulin resistance in adipocytes of lean females and whether the same response is seen in the adipocytes of obese females are unknown.

Foxo1 and forkhead box A1 (foxa1) are regulators of adipocyte metabolism located downstream of the insulin receptor[10], and function as co-regulators of classical, nuclear AR action[11]. Foxo1, an inhibitor of AR transcriptional activity, is phosphorylated downstream of insulin receptor substrate 2 (IRS2) in the insulin signaling pathway, whereas foxa1, an activator of AR transcriptional activity, is phosphorylated downstream of both insulin receptor substrate 1 (IRS1) and IRS2[12]. The function of the forkhead box family of proteins is modulated through phosphorylation by protein kinase B (Akt) among other kinases[13]. In preliminary studies conducted in the Newell-Fugate lab, it was found that testosterone administration to intact, lean female pigs over a course of 14 days resulted in down-regulation of phosphorylated Akt, a member of the insulin signaling pathway, in visceral but not in subcutaneous fat. This discovery suggests that androgens themselves may play a role in the promotion of insulin resistance in females.

The objective of this research is to identify whether androgens regulate insulin signaling in induced murine 3T3-L1 adipocytes. The central hypothesis is that androgens, independent from obesity, cause disruption of insulin signaling in adipocytes at the level of the AR.

Specifically, we hypothesize that androgens down-regulate the insulin signaling pathway directly via classical stimulation of the genomic AR and cause decreased glucose uptake in adipocytes. This central hypothesis will be tested, and the objective obtained, by examination of one specific aim: Assessment of the effects of androgens on insulin signaling and glucose uptake in induced murine 3T3-L1 adipocytes.

SECTION II

METHODS

A. Cell culture

3T3-L1 pre-adipocytes (ATCC, Manassas, VA) were seeded onto 100x20 mm tissue culture (TC)-treated dishes (Costar, Corning, Corning, NY) with DMEM/F12 (Gibco, Invitrogen, Carlsbad, CA) base medium containing 10% FBS (VWR, Radnor, PA) and 1% antibacterial/antifungal cocktail (100x Anti-Anti, Gibco, Invitrogen). The 3T3-L1 pre-adipocytes were grown to about 70% confluency, and were then passaged at a density of 70,000 live cells/well in two TC-treated 6-well plates (Costar, Corning) and two 60x20 mm TC-treated dishes (Costar, Corning), and grown to 100% confluency. Forty eight hours after the cells reached confluency and became over-confluent, they were treated for 48 hours with adipocyte differentiation medium, composed of DMEM/F12 base medium with 1.0 µM dexamethasone (G-biosciences, St. Louis, MO), 0.5 mM IBMX (Sigma-Aldrich, St. Louis, MO), and 1.0 µg/mL insulin (Sigma-Aldrich). Following the treatment with adipocyte differentiation medium, cells were treated with adipocyte maintenance medium, composed of DMEM/F12 base medium with 1.0 µg/mL insulin, for seven days or until they were 60- 80 % differentiated into adipocytes. Differentiation was assessed qualitatively by staining the two dishes with Oil Red O (Sigma-Aldrich) and Mayer's hematoxylin (Electron Microscopy Sciences, Hatfield, PA). Cells were then treated for 48 hours with either control ethanol (Koptec, Decon Labs, King of Prussia, PA) vehicle or DHT (10^{-6} M in ethanol vehicle; Steraloids, Newport, RI) in adipocyte maintenance media. Treatments were administered in duplicate or triplicate for technical replication. After 48 hours, the media from each well was

collected. Cells from Plate 1 were lysed with 950 µL 10X RIPA lysis buffer (Cell Signaling Technologies, Boston, MA) for protein extraction. Cells from Plate 2 were lysed with 1 mL TRIzol reagent (Invitrogen) for mRNA extraction. The media and contents of each lysate were stored in a 1.5 mL DNase/RNase free snap cap tube (Fisherbrand, Hampshire, NH) at -80°C until further analysis.

B. RNA extraction and quantitation, cDNA synthesis, protein quantitation, and real time PCR

Two hundred microliters of chloroform was added to each of the TRIzol-treated lysates, which were then vortexed, and centrifuged at 12,000 g for 15 minutes at 4°C. The upper aqueous phase was transferred into new DNase/RNase free snap cap tubes, and 0.5 mL of isopropyl alcohol were added to the tubes in order to precipitate the RNA. The tubes were centrifuged at 12,000 g for 10 minutes at 4°C, and after removing the supernatant, the pellets were washed with 1 mL of 75% ethanol. The samples were then centrifuged at 7,500 g or 5 minutes at 4°C. After removing excess ethanol, the RNA pellets were allowed to dry for 10 minutes before being dissolved in 10 µL of water treated with DEPC (Sigma-Aldrich). After incubating the samples on a 55°C heat block, they were stored in the -80 freezer for later use. Isolated RNA was diluted 1:1000 using 1 µL of the concentrated RNA pellets and 999 µL of DEPC-treated water. After creating a calibration curve using two pre-prepared standards, 1 µL of the RNA dilutions and 199 µL of Qubit working solution (Invitrogen) were mixed and ran on the Qubit 3.0 Fluorometer in order to determine RNA concentration. Protein concentration was determined by diluting protein samples 1:25 using DEPC-treated water and running the samples on a Qubit 3.0 Fluorometer using a Qubit Protein Assay Kit (Invitrogen). Reverse

transcription was performed on the RNA in order to convert it into cDNA for PCR analysis, using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and an iCycler (Bio-Rad Laboratories, Hercules, CA) for temperature fluctuation. RNA transcript expression for forkhead box 1 (FOXO1), insulin receptor (INSR), and protein kinase B isoform 2 (Akt2) relative to housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH), was assessed using Taqman-based real time PCR.

C. Glucose determination in media

The amount of glucose (mg/dL) in the media from each well was determined using the Glucose Colorimetric Detection Kit (Arbor Asssays, Ann Arbor, MI). Media samples were thawed and diluted to a 1:10 ratio using PBS. Twenty microliters of each of the 7 glucose standards were pipetted into a 96 well plate (Costar, Corning). Twenty microliters of assay buffer, 25 µL of HRP solution, 25 µL of Calorimetric solution, and 25 µL of glucose oxidase concentrate solution were added to the wells following the addition of the glucose samples and standards. The solutions were incubated at room temperature for 30 minutes before being read with a BioTek Gen5 microplate reader at 560 nm.

D. Statistical analysis

All data was assessed for normality using PROC UNIVARIATE (SAS, Inc, Cary, NC). Non-normal variables were logarithmically transformed prior to comparison of treatments using un-paired Student's T-test. Results are displayed as least squares mean (LSM) ± standard deviation (SD). The level of significance was set at p=0.05.

SECTION III

RESULTS

A. Real time PCR Analysis:

There was no significant difference in RNA transcript expression of FOXO1, INSR, and Akt2 relative to GAPDH between control and DHT-treated 3T3-L1 induced adipocytes (p=0.9; Figure 2). FOXA1 was also assessed but was unable to be detected in any of the treated cells.

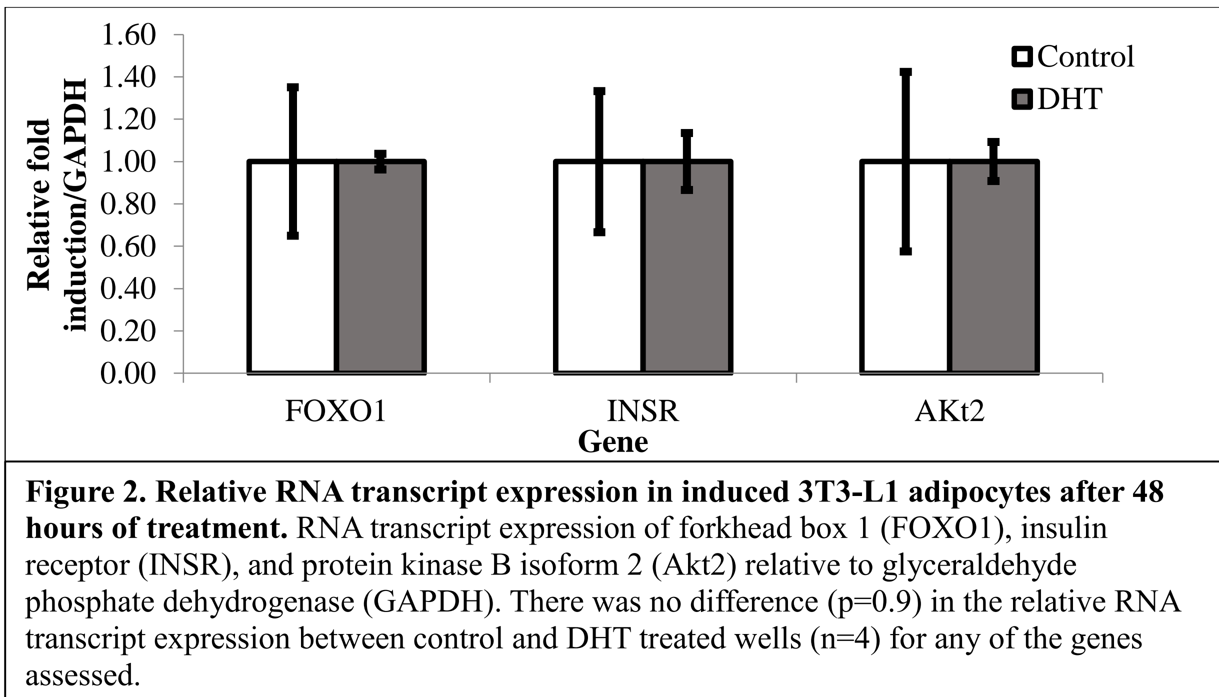

Figure 2. Relative RNA transcript expression in induced 3T3-L1 adipocytes after 48 hours of treatment. RNA transcript expression of forkhead box 1 (FOXO1), insulin receptor (INSR), and protein kinase B isoform 2 (Akt2) relative to glyceraldehyde phosphate dehydrogenase (GAPDH). There was no difference (p=0.9) in the relative RNA transcript expression between control and DHT treated wells (n=4) for any of the genes assessed.

B. Glucose Concentration in Cell Culture Media

i. RNA quantitation:

RNA content in control wells was 52.8 ng (C1) and 26.8 ng (C2), respectively. RNA content in DHT-treated wells were 50.9 ng (DHT1) and 42.4 ng (DHT2), respectively.

Glucose found in the cell culture media was standardized to RNA (ng) per well (C1=0.219 mg glucose/ng RNA;, C2=0.858 mg glucose/ng RNA); DHT1=0.517 mg glucose/ng RNA; DHT2=0.246 mg glucose/ng RNA). The control wells had 0.575 ± 0.401 mg glucose/ ng RNA. The DHT-treated wells had an 0.381 ± 0.191 mg glucose/ ng RNA (Figure 1). There was no significant difference in the amount of glucose found in the media of control versus DHT treated wells when calculated using RNA quantity in the wells (p>0.601).

ii. Protein quantitation:

Protein content in control wells was 2700 µg (C3) and 1590 µg (C4), respectively. Protein content in DHT-treated wells were 2120 µg (DHT3) and 1630 µg (DHT4), respectively. Glucose found in the culture media was standardized to protein (µg) per well (C3=0.007 mg glucose/ µg protein; C4=0.036 mg glucose/ µg protein; DHT3=0.018 mg glucose/ µg protein; DHT4=0.032 mg glucose/ µg protein). The control wells had 0.022 ± 0.021 mg glucose/ µg protein. The DHT-treated wells had 0.025 ± 0.010 mg glucose/ µg protein (Figure 1). There was no significant difference in the amount of glucose found in the media of control versus DHT-treated wells when calculated using protein quantity in the wells (p>0.849).

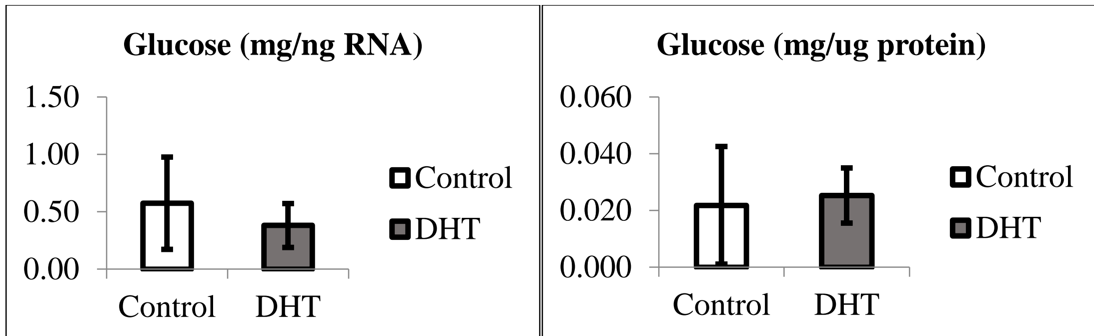

Figure 1. Glucose concentration in media from induced 3T3-L1 adipocytes after 48 hours of treatment. A. Glucose concentration (mg glucose/ng RNA) in control or DHT treated wells collected for PCR analysis (n=4). **B.** Glucose concentration (mg glucose/µg protein) in control or DHT treated wells collected for protein analysis (n=4). There was no difference (p>0.6) in glucose concentration between control and DHT treated wells for either experiment.

SECTION IV

CONCLUSION

The results of this study imply that further experimentation will be necessary in order to determine the role the androgen DHT has on insulin signaling in induced 3T3-L1 adipocytes. The addition of DHT did not alter the glucose concentration in the media of induced 3T3-L1 adipocytes, as shown by the large p-values. There was also no difference in RNA transcript expression of FOXO1, INSR, Akt2 in induced 3T3-L1 adipocytes between control and DHT-treated wells. We were unable to detect FOXA1, implying its role in regulating the androgen receptor in 3T3-L1 pre-adipocytes may be limited following differentiation.

Although we did not detect differences between control and DHT treated wells in any parameter assessed, we only completed one replicate of the experiment and only a few technical replicates (i.e., treated wells) per treatment. Therefore, our power to detect differences between the treatments was very low. It is also possible that DHT does not alter insulin signaling in 3T3-L1 cells. It may prove useful to investigate ways to estimate the number of mature, live adipocytes in each well following differentiation in order to provide a more reliable measure of glucose concentration within the media. Oil Red O staining on its own only provided a crude estimate of the percentage of cells that successfully underwent differentiation, and could only account for the dishes stained. Additionally, protein analysis via western blotting would be helpful for evaluating the expression of proteins involved in the insulin signaling pathway. Further replication of the experiment to increase the power is recommended.

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