THE ROLE OF UNCOUPLING PROTEIN 2 IN THE DEVELOPMENT OF INSULIN REISTANCE IN 3T3-L1 ADIPOCYTES

An Honors Fellows Thesis

by

DAVID AUREL GOODWIN JR

Submitted to the Honors Programs Office
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

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Major: Chemical Engineering

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Approved by:

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ABSTRACT

The Role of Uncoupling Protein 2 in the Development of Insulin Resistance in 3T3-L1 Adipocytes. (April 2010)

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Type 2 Diabetes is becoming a major health concern throughout the world. Recent clinical evidence points to restoring overall insulin sensitivity as the major objective in the management of Type 2 Diabetes. Current research indicates Uncoupling Protein 2 (UCP2) may play a role in the development of insulin resistance and the onset of Type 2 Diabetes. UCP2 over expression has been linked to increases in reactive oxygen species concentration. The presence of UCP2 has been linked to the ability of the cell to perform insulin-stimulated uptake of glucose. Tumor Necrosis Factor α has also been linked to increases in ROS concentration and the insulin sensitivity of the cell. This evidence suggests a link between UCP2, ROS, TNF-α, and insulin resistance. The overall objective of our research is to establish the relationship between UCP2 and ROS in the context of insulin resistance in 3T3-L1 adipocytes with the specific aim of determining the effect of UCP2 expression on ROS concentration. To date we have succeeded in cloning the UCP2 gene into an inducible plasmid, pRev-TRE, inducible by the addition

of Doxycycline. The development of a retroviral vector, able to integrate the pRev-TRE UCP2 plasmid into the genome of 3T3-L1 adipocytes, is currently underway. Our future work will include experiments quantifying the concentration of ROS in the presence of various levels of UCP2 expression.

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NOMENCLATURE

Dox	Doxycycline
FFA	Free Fatty Acid
ROS	Reactive Oxygen Species
TNF-α	Tumor Necrosis Factor α
UCP2	Uncoupling Protein 2

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CHAPTER I

INTRODUCTION

Introduction

Type 2 Diabetes is quickly becoming one of the most prominent diseases of the developed world. The 171 million estimated cases in 2000 are expected to increase to at least 366 million by 2030. An even more negative statistic is the increasing number of adolescents that develop the disease, which was once only associated with adults [1]. The disease is also becoming more prevalent in the developing world where obesity has increased from 2.3 to 19.6% over the last decade [2].

Type 2 Diabetes is characterized by the gradual development of the resistance to the action of insulin, a relative decrease in insulin secretion, and elevated plasma levels of free fatty acids and glucose. A number of abnormalities, collectively known as the metabolic syndrome, are associated with the development of Type 2 diabetes including: central obesity, dyslipidemia, hyperinsulinemia, elevated plasma inflammatory markers, diminished plasma Acrp30 levels, impaired fibrinolysis, vascular abnormalities and hypertension [3].

This thesis follows the style of *Molecular Therapy*.

Anti-diabetic medications aimed at lowering plasma glucose levels have proven ineffective at restoring metabolic homeostasis and preventing the progression of Type 2 diabetes. However new methods of treatment with insulin-sensitizing compounds, aimed at improving insulin sensitivity, have been shown to restore metabolic homeostasis and improve the abnormalities associated with metabolic syndrome. This evidence indicates the importance of restoring overall insulin sensitivity in the clinical management of Type 2 diabetes [3].

Background

Uncoupling protein 2 (UCP2) belongs to a family of five homologous uncoupling proteins, UCP1-UCP5. UCP 2 mRNA is found in a wide variety of tissues including the spleen, pancreatic β-cells, heart, lungs, brown adipose and white adipose. However protein expression is not proportional to mRNA concentration and has not been detected in several of the tissues that contain mRNA at relatively high concentrations. Also the relative change in concentration of mRNA does necessarily predict changes in the expression of protein [4]. Though not conclusively determined, by virtue of UCP2's location within the inner mitochondrial matrix it has been suggested that UCP2 may have a physiological function relating to the metabolism of the cell [5].

Many possible physiological functions for UCP2 are currently being investigated including a role for UCP2 in the defense against reactive oxygen species and prevention of oxidative damage. Cell metabolism utilizes the proton gradient generated when protons are actively pumped into the inner mitochondrial membrane to synthesize ATP.

However, as the potential across the membrane increases, the production of reactive oxygen species (ROS) also increases. A proposed mechanism suggests that UCP2 acts as a proton leak across the inner mitochondrial matrix, therefore reducing the mitochondrial membrane potential and preventing sustained production of ROS [6].

Experimental evidence also supports this theory, as it has been shown that metformin-induced oxidative stress in the 3T3-L1 adipocytes results in the over-expression of UCP2 (i.e., a feed-forward mechanism) [7]. Other experiments have demonstrated a link between physiological concentrations of mono-and poly-saturated dietary fatty acids and the level of UCP2 mRNA in 3T3-L1 pre-adipocytes. These studies also show that the increase in UCP2 mRNA is a result of increased transcription of the UCP2 gene rather than increased stability of the existing mRNA or protein [8].

UCP2 function has also been linked to insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Insulin-stimulated glucose uptake was measured in the presence of an UCP2 inhibitor, genipin, and compared to values obtained without the inhibitor. The presence of genipin caused a notable decrease in glucose uptake, therefore linking UCP2 activity to insulin sensitivity [9].

Recent research has also linked tumor necrosis factor α to insulin resistance in adipose tissue. It has been shown that long-term exposure to TNF-α induces insulin resistance and neutralization of TNF-α increases insulin sensitivity and reduces hyperinsulinemia [3]. This is of particular interest to the current discussion because TNF-α also acts as a

signal in the apoptotic pathway and the presence of ROS is known to induce apoptosis by causing the mitochondrion to expel cytochrome c.

Research plan

The overall objective of the proposed research is to establish the relationship between UCP2 and ROS in the context of insulin resistance in 3T3-L1 adipocytes.

Specific aim

Determine the effect of UCP2 expression on ROS levels and TNF-α production in 3T3-L1 adipocytes.

Hypothesis 1

Forced expression of UCP2 increases insulin sensitivity through decrease in oxidative stress

CHAPTER II

METHODS

Cell culture

3T3-L1 preadipocytes (ATCC, VA) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin. 293-T cells for retrovirus production were grown in high-glucose DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air [10].

Production of pRevTRE-UCP 2 plasmid

The starting plasmid for UCP2 expression, pRevTRE-XMCS, was generated by inserting the multiple cloning site sequence, XMCS, into a previously developed pRevTRE plasmid via ligation. This new plasmid, pRevTRE-XMCS, was electroporated into *E. coli* and transformants isolated. Colonies were selected and the fidelity of the cloned plasmid validated through multiple restriction digests and sequencing. Using a similar method, the UCP2 gene was then introduced into the XMCS plasmid.

Production of virus containing UCP2

The lentiviral backbone containing pRevTRE-UCP2, encoding HIV-1 gag-pol, HIV-1 and VSV-G envelope was cotransfected into 293-T cells using the calcium phosphate procedure optimized for 293T cells. Fresh medium was added at 24 hours and the virus collected at 48 hours [10].

Viral transduction

Viral supernatant was diluted with fresh medium and supplemented with 5 μg/ml of Polybrene before adding to the cells. The viral supernatant solution was replaced with fresh medium at 48 hours and the results analyzed after 96 hours [10].

Antibiotic selection of cells transduced with UCP 2 virus

The plasmid containing the UCP2 gene was inserted into the genome of the 3T3-L1 adipocytes using the retroviral transduction procedure described previously and a dilution of 10^{-2}. The viral supernatant was replaced with fresh medium supplemented with 200 μg/mL hygromycin-b. Fresh medium containing hygromycin-b was added every other day, and the cells monitored for antibiotic resistant colonies.

Determination of viral functionality

The functionality of the retroviral product was determined using rtPCR to amplify UCP2 mRNA recovered from the isolation of RNA in 3T3-L1 adipocytes.

Manipulation of UCP2 expression

The plasmid containing the UCP2 gene was inserted into the genome of the 3T3-L1 adipocytes using the retroviral transduction procedure described previously. UCP2 expression was activated by addition of doxycyline at a concentration of 1 μg/mL. The UCP2 inhibitor genipin was used as a negative control as it allowed the effect of UCP2 expression without any baseline activation [9].

Manipulation of ROS concentration

ROS was induced in 3T3-L1 adipocytes by two methods. Metformin, which causes an increase in ROS production by binding to complex 1 of the respiratory chain, thus preventing electron transfer [7], was used as one method while the exogenous addition of polyunsaturated fatty acids (e.g., linoleic acid) was used as it has also been shown to increase ROS production in 3T3-L1 adipocytes. External addition of hydrogen peroxide was used as a positive control.

Quantifying UCP2

The levels of UCP2 protein in 3T3-L1 adipocytes was determined by Western blots. In order to facilitate detection, mitochondria were isolated from 3T3-L1 cells, so as to reduce the complexity of the protein sample and enriching the levels of UCP2 [7].

Quantifying ROS and TNF-α

Intracellular ROS concentration was measured using the dihydroethidium (DHE) fluorescence/HPLC assay [11]. The expression of TNF-α was tracked using commercially available ELISa kits (R&D Systems, MN) [8].

Quantifying insulin-stimulated uptake of glucose

3T3-L1 cells were starved in serum-free DMEM containing glucose for two hours, after which these cells were washed and placed in KRBH buffer. The cells were be incubated either in the presence of insulin or without insulin followed by the addition of 2-DG and then washed and again placed in KRBH buffer. The cells were then lysed and protein content measured by the BCA method [9].

CHAPTER III

RESULTS

Production of the pRevTRE-UCP 2 plasmid

The gene coding for UCP2 was successfully cloned into an inducible plasmid system (pRev-TRE, Clontech, CA). In this system, addition of an inducer (doxycycline) leads to activation of a trans-activator protein, which then binds and induces expression of the target gene.

The starting plasmid for UCP2 expression, pRevTRE-XMCS, was generated by inserting a synthetically assembled multiple cloning site sequence, XMCS, into the previously developed pRevTRE plasmid via ligation. This new plasmid, pRevTRE-XMCS, was electroporated into *E. coli* and transformants isolated. Colonies were selected and the fidelity of the cloned plasmid validated through multiple restriction digests and sequencing. Using a similar method, the UCP2 gene was then introduced into the XMCS plasmid.

Production of virus containing the pRevTRE-UCP 2 plasmid

Viral transduction was chosen as the means for inserting the pRevTRE-UCP 2 plasmid into the genome of 3T3-L1 adipocyte cells because the viral mechanism is more consistent (uniform number of plasmids per cell) and efficient than electroporation.

Three methods of multiple plasmid cell transfection were analyzed to determine which reagent would most efficiently transfect 293T cells. The methods tested were GenJet transfection optimized for Hep-G2 cells, Fugene transfection optimized for multiple plasmid transfection, and calcium phosphate transfection optimized for 293T transfection. It was determined that calcium phosphate transfection was the most efficient and reliable transfection method, consistently giving 90-95% transfection rates.

Calcium phosphate transfection protocol was followed to introduce the pRevTRE-UCP2 plasmid into the 293T cells. This transfection procedure involved preparing a solution of calcium dichloride, HBS, encoding HIV-1 gag-pol plasmid, HIV-1 and VSV-G envelope plasmid, and pRevTRE-UCP2 plasmid. This solution formed a precipitate, containing the three plasmids, that was able to diffuse across the 293T cell membrane. Five minutes before adding the plasmid solution to the 293T cells, chloroquine was added. The cells were then exposed to the chloroquine and plasmid solution for 24 hours. The cells supernatant was then replaced by fresh media containing 10 mM sodium butyrate and incubated for eight hours, after which the supernatant was replaced with fresh media. The virus was collected on the third day and every 48 hours after, over the course of six days. A change in pH, as indicated by the color change from red to yellow, suggested the presence of virus in the 293T supernatant.

The pRevTRE-UCP 2 plasmid to be inserted by the retrovirus also provides the cell antibiotic resistance to hygromycin-b. By subjecting the transfected cells to media supplemented with hygromycin-b, the functionality of the virus can be tested and a preliminary viral titer obtained. Single colonies were observed in a viral transduction at a one to one hundred dilution of virus to media suggesting the viral titer in recovered media is quite low.

The functionality of the virus was further tested by transducing Hela pTeton cells with the recovered pRevTRE-UCP 2 retroviral product and amplifying recovered RNA using a rtPCR reaction with UCP 2 primers. The results were confirmed using gel electrophoresis and are seen in the following Figures 1-3.

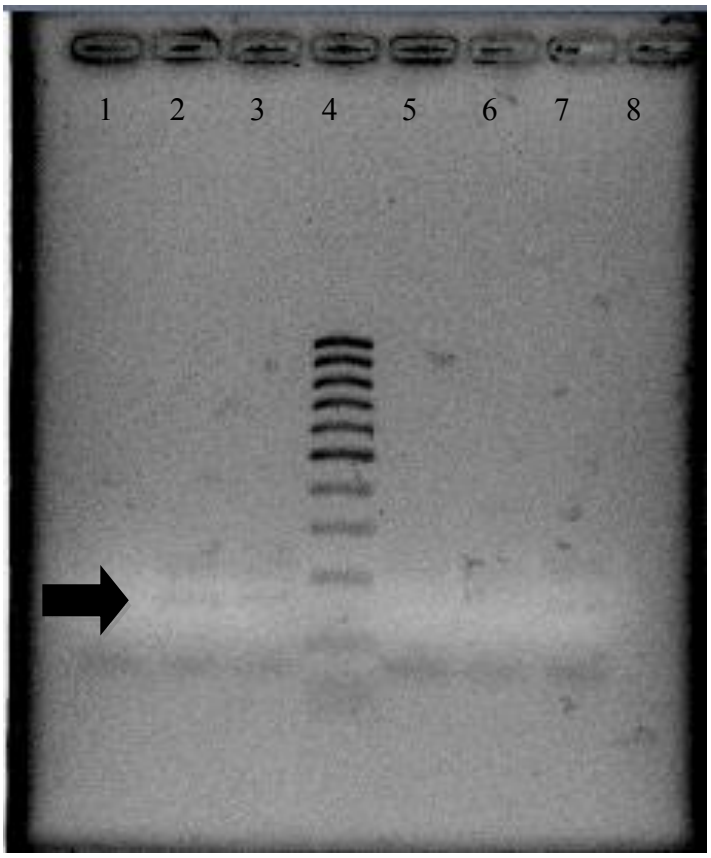

Figure 1. First DNA gel showing the products of a PCR amplification, using UCP 2 primers, of RNA recovered from Hela pRevTRE-pTeton cells transduced with UCP 2 virus. Wells one and two contain mRNA recovered from cells exposed to a one-third dilution of virus. Wells three and five contain mRNA recovered from cells exposed to one sixth dilution of virus. Wells six and seven contain mRNA recovered from cells not exposed to virus.

The UCP 2 mRNA amplicon is approximately 180 base pairs long; therefore, if the virus was functioning properly, a strong band just below the 200 base pairs would be present in cells exposed to the virus and no band in the cells not exposed to virus. However, Figure 1 shows a faint band at 180 in wells 2, 3, and 7. A second experiment was conducted to clarify and the results are seen in Figure 3.

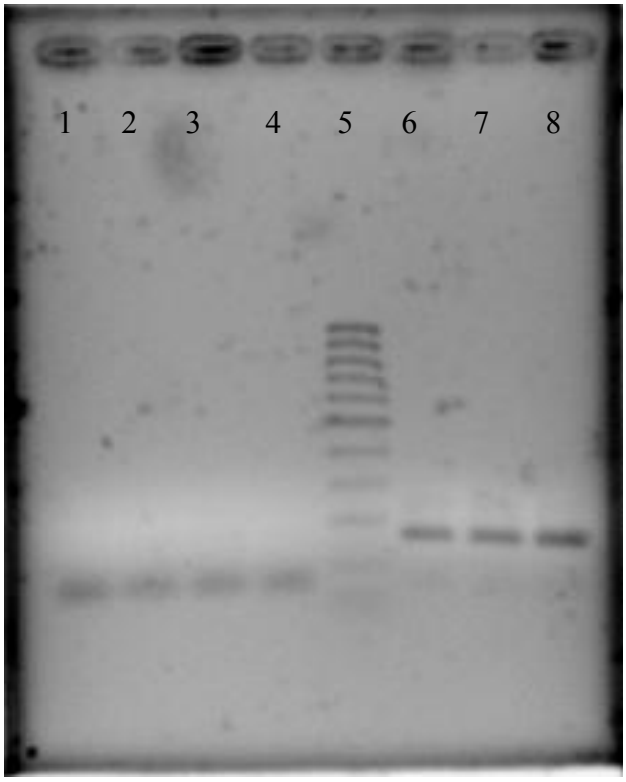

Figure 2. Second DNA gel showing the products of a PCR amplification, using UCP 2 primers, of RNA recovered from Hela pRevTRE-pTeton cells transduced with UCP 2 virus. Wells one through four contain mRNA recovered from cells exposed to a one sixth dilution of virus. Wells six, seven, and eight contain mRNA recovered from cells not exposed to virus.

The gel shown in Figure 2 also shows ambiguous results. Wells one through four were exposed to virus but do not show a band at 180 base pairs, where as wells six through eight were not exposed to virus and have a strong band at 180 base pairs. A third experiment was conducted to optimize annealing temperature of the PCR reaction, and a fourth experiment was performed using the new annealing temperature. The results are shown in Figure 3.

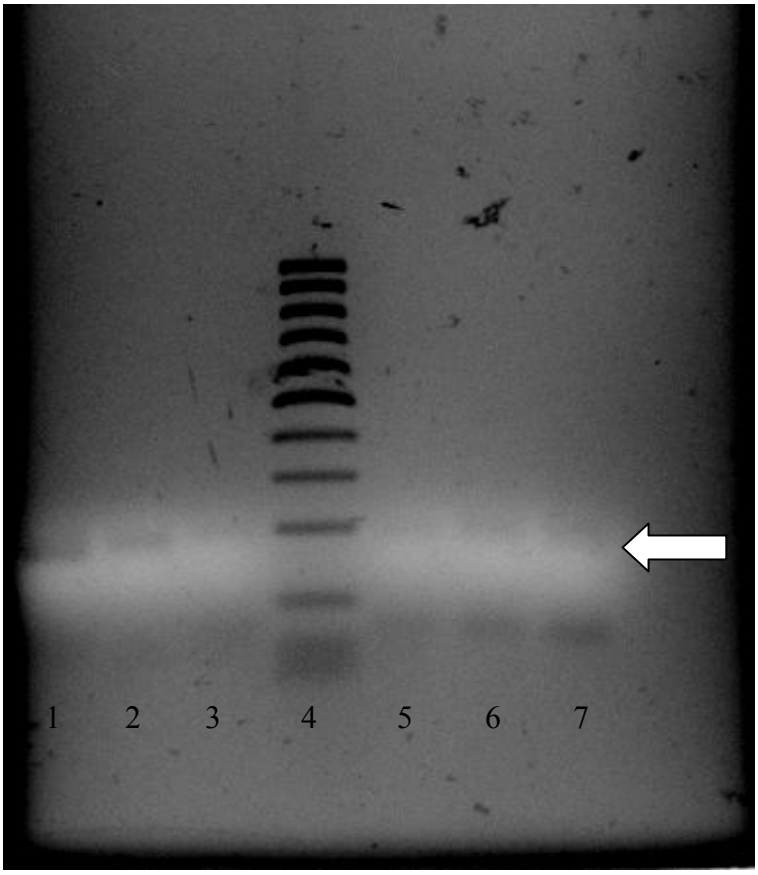

Figure 3. Third DNA gel showing the products of a PCR amplification, using UCP 2 primers, of RNA recovered from Hela pRevTRE-pTeton cells transduced with UCP 2 virus. Wells one and two contain mRNA recovered from cells exposed to a one-sixth dilution of virus. Wells three and five contain mRNA recovered from cells exposed to one sixth dilution of virus. Wells six and seven contain mRNA recovered from cells not exposed to virus.

Wells one, two, three, and five contain RNA extracted from cells exposed to UCP 2 retrovirus and each shows a band at 180 base pairs. Wells six and seven contain RNA from cells not exposed to virus and also have a band at 180 base pairs. These results combined with the antibiotic selection results suggest that a functional virus was recovered at very low concentrations, and that UCP 2 is naturally expressed in Hela cells.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Though the results of the experiments suggest that a functional virus is being produced at low concentrations, the inconsistency of the data indicates the process of viral production needs to be optimized. Currently the virus is being collected and stored in one ml aliquots at -80°C. The retroviral product is produced at low concentrations and is inconsistent among the samples collected. This inconsistency in viral titer could explain why cells exposed to the same dilution of virus are not showing similar results. Steps should be taken to concentrate the virus using ultra-centrifugation or PEG precipitation so that a high titer virus preparation can be used in all experiments.

Recent research has also indicated that transgenes (i.e., genes over-expressed through integration of a plasmid) that are over-expressed are subject to higher rates of degradation and removal from cells than genes expressed at low copy numbers. This suggests the experimental design should be modified to first optimize the concentration of dox used (which directly influences the level of UCP2 expression) and the resultant UCP2 expression.

Once viral production and cell stimulation have been optimized colonies of 3T3-L1 adipocyte pRevTRE-pTeton-UCP 2 cells can be selected and propagated. Assuming integration of the retroviral plasmid does not affect differentiation, the relationship between UCP2 and ROS in the context of insulin resistance in 3T3-L1 adipocytes can be

investigated using the experiments outlined in the preceding paragraph and the methods described in chapter 2.

Future experiments

The relationship between UCP2 and ROS in the context of insulin resistance in 3T3-L1 adipocytes will be investigated using the four experiments described below.

Experiment outline 1

Measurements of ROS and TNF-α will be taken for 3T3-L1 cells under baseline physiological, blank and DOX activated UCP2 conditions.

Experiment outline 2

Measurements of UCP2 and TNF-α will be taken for 3T3-L1 cells under baseline physiological and ROS induced conditions.

Experiment outline 3

Measurements of UCP2, ROS and TNF-α will be taken for 3T3-L1 cells at varying concentrations of glucose and FFA in the culture medium.

Experiment outline 4

Measurements of insulin-stimulated glucose uptake will be taken at baseline physiological, blank and DOX activated UCP2 levels. Also at baseline physiological and increased levels of ROS.

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