GENE EXPRESSION PROFILING OF 3T3-L1 ADIPOCYTES EXPRESSING THE MITOCHONDRIAL UNCOUPLING PROTEIN 1 (UCP1)

A Senior Scholars Thesis

by

FATIH S. SENOCAK

Submitted to the Office of Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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ABSTRACT

Gene Expression Profiling of 3T3-L1 Adipocytes Expressing the

Mitochondrial Uncoupling Protein 1 (UCP1) (April 2006)

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During the past 20 years, there has been a significant increase in the number of individuals developing type II diabetes mellitus (T2DM). Evidence from several studies indicates that obesity and weight gain (increase in white adipose tissue) are associated with an increased risk of developing diabetes. Current treatments to combat this epidemic involve the reduction in white adipose tissue (WAT). Previously we proposed that the forced expression of uncoupling protein 1 (UCP1), normally part of the thermogenic mechanisms found in brown adipose tissue (BAT), can be used

to reduce accumulation of triglycerides in white adipocytes, and thereby regulate body fat mass. The aim of this study was to determine the effects of forced UCP1 expression on global changes in energy metabolism in white adipocytes. Specifically, we used DNA microarrays to characterize the changes in white adipocyte gene expression upon UCP1 expression and determine the extent to which UCP1 expressing white adipocytes emulate brown adipocytes. Murine 3T3-L1 preadipocytes, either expressing UCP1 or control (i.e., no UCP1) were cultured to confluence. On day 2 post confluence, the preadipocytes were induced to differentiate using a standard adipogenic cocktail consisting of insulin, isobutylmetyhylxanthine (IBMX), and dexamethasone (DEX). At 10 days post-isolation, total RNA was isolated and the transcript levels profiled using the Codelink microarray system (Agilent, CA).

DEDICATION

To my Parents.

ACKNOWLEDGMENTS

I thank Dr. Arul Jayaraman for his immense support and encouragement towards pursuing my interest in scientific research. He has given me tremendous amount of guidance without which I could not have accomplished my research goals.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION[1]

During the last 20 years, obesity has risen significantly in the United States. (1). Based on the current statistics, it is believed that 30% of Americans are obese and an additional 35% of Americans are overweight making 65% of the U.S. population carrying excess weight. In addition, it is not only the adults that are carrying excess weight but the numbers for obese children and teens between the ages of 6-15 have tripled within the same time period. Because of this growing epidemic, the demand for treatment options has become increasingly necessary.

Obesity is defined as a chronic condition that develops due to excess accumulation of fats in adipose tissue and skeletal muscle. It raises the risk for many diseases ranging from type II diabetes, hypertension, coronary heart disease, and cancer. Currently, there are only limited methods for treating obesity. Majority of the treatments involve diet and lifestyle modification. At this time, only two FDA approved drugs (siburtramine and

[1] This thesis follows the style and format of *The Journal of Biological Chemistry.*

orlisat) are available. (2) However, these drugs have had little success as there are numerous side effects.

Within the last few years, the view of adipose tissue has changed from that of a "mere energy store or provider of thermal and mechanical insulation". (3) Current research has shown adipose tissue to have important endocrine functions that are vital for the maintenance of the body's homeostatis. Several of the hormones and signaling factors secreted by adipose tissue include Leptin, Lipoprotein lipase and several inflammatory cytokines. These newly discovered endocrine functions of adipose tissue now have led researchers to classify obesity as a state of chronic inflammation due to the observed elevated plasma levels of inflammatory markers such as interleukin (IL)-6, Tumor necrosis factor (TNFα) or C-reactive protein (CRP) (4).

Besides its endocrine functions, WAT also contains anabolic and catabolic reactions as part of its metabolic functions. Lipogenesis uses Acetyl-Coenzyme A (CoA) subunits to synthesize fatty acids. In turn, triacylglycerides are synthesized by esterification of the fatty acids to

glycerol-3-phosphate. ß-oxidation, the opposing catabolic reaction to lipogenesis, breaks down triacylglycerides into Acetyl-CoA units for the cell to use as fuel in the mitochondria.

It has been demonstrated that two types of adipose tissues exist: white adipose tissue (WAT), which stores triglycerides, and brown adipose tissue (BAT) which is specialized for adaptive non-shivering thermogenesis. (5) BAT is only found in newborns and children and begins to disappear as the individual grows and only WAT is found in adult humans. Therefore, understanding the biology of WAT is important for developing therapeutic strategies for obesity and related complications.

In eukaryotic cells, mitochondria are considered to be the "powerhouse of the cell" due to their ability to generate the majority to the ATP needed for the cell to survive. Mitochondria, a bilipid organelle, achieve this through a process referred to as oxidative phosphorylation. Cellular respiration, the process of oxidizing different molecules to generate ATP, occurs in the mitochondria. Mitochondria contain an inner membrane (cristae) and an outer membrane. The inner membrane contains five integral proteins,

Complex I (NADH dehydrogenase), Complex II (Succinate Dehydrogenase), Complex III (Cytochrome C reductase), Complex IV (Cytochrome C oxidase) and Complex V (F1-F0 ATP Synthase). Using the cofactors NADH and FADH2, the complexes pump protons into intermembrane space of the mitochondria to create a proton gradient. In order to generate ATP, these protons flow through the F1-F0 ATP synthase.

In BAT, UCP1 is believed to integrate itself into the mitochondrial membrane causing the hydrogen ions to leak prematurely. The loss of the proton gradient generates heat and reduces ATP synthesis. Because of this, the cell looks to other metabolic pathways to makeup for the net loss in ATP production. This function for UCP1 makes it a promising candidate for WAT reduction for obesity treatment.

CHAPTER II

PROBLEM

Hypothesis

Forced UCP1 expression in white adipocytes will alter the cells expression profile such that it will emulate the thermogenic phenotype of brown adipocytes.

Specific Aim1

To determine the effects of forced UCP1 expression on global changes in energy metabolism in white adipocytes. Specifically, to use DNA microarrays to characterize the changes in white adipocyte gene expression upon UCP1 expression and determine the extent to which UCP1 expressing white adipocytes emulate brown adipocytes.

Specific Aim2

To clone Uncoupling Protein 2 plasmid construct for future studies of uncoupling protein biochemistry in white adipocytes.

Problem Summary

The purpose of this study is to characterize the metabolic pathways altered in white adipocytes due to the forced expression of UCP1.

CHAPTER III

METHODS

Cell Culture

Previously transfected murine 3T3-L1 preadipocytes with pRevTre and pRevTre-UCP1 plasmids will be cultured in Dulbecco's Modified Eagle Medium (DMEM) purchased from Invitrogen-Gibco (Carlsbad, California). DMEM will contain will be supplemented with CS (10 % v/v), penicillin (200 u/ml) and streptomycin (200 µg/ml). Cells will be maintained in an incubator at 37 °C, with 10% Carbon Dioxide in a fully humidified atmosphere. During this period, medium was replenished every other day. On day 2 post-confluence, the cells were induced to differentiate in an adipocytes medium (DMEM with 10%FBS and penicillin/streptomycin) supplemented with a standard adipogenic medium cocktail consisting of 1ug/ml insulin, 0.5 mM isobutylmethylxanthine (IBMX) and 1µM dexamethasone (DEX). After 48 hrs, the first induction medium was replaced with a second induction medium consisting of the basal adipocytes medium supplemented with only insulin. After another 40 hrs, the second induction medium was replaced

with the basal adipocytes medium. Medium was replenished every other day through day 10 post induction.

RNA Extraction

At 10 days post-isolation, total RNA was isolated using the RNeasy Mini Kit (Qiagen, CA) and the transcript levels profiled using the Codelink microarray system (Agilent, CA).

Bioinformatic Analysis of Microarray Data

Triplicate samples were used for cell type and one array was used for each sample. The raw data was initially was filtered by selecting only those genes that had a "G(ood)" quality flag. Afterwards a T-test was performed ($p<0.05$) to determine the significant change in the expression levels between control and UCP1 adipocytes. Only those genes that passed the G(ood) quality flag and the T-Test was chosen for further analysis.

Construction of RevTRE-UCP2 Vector

The pRevTET-Off, pRevTRE vectors were purchased from BD Biosciences (Mountain View, CA). Plasmid pCMV-Sport6-UCP2 containing the mus musculus full length cDNA for UCP2 was purchased from Invitrogen (Carlsbad, CA). It was digested with Sal1 and Xba1 to remove the UCP2 gene from the plasmid. pSP72, a cloning vector was purchased from Promega (Madison, WI). It was digested with Sal1 and Xba1 and then ligated to the UCP2 gene cut from pCMV-Sport6-UCP2. pSP72 was then digested with Sal1 and Cla1 to remove UCP2 which was further ligated into pRevTRE, digested with the same set of restriction enzymes.

CHAPTER IV

RESULTS

To identify the effects of UCP1 expression on metabolic functions of white adipocytes, all those genes coding for enzymes in a metabolic processes were examined.

Table 1: Metabolic pathway gene expression changes

Glycolysis	Down regulated
Glycerogenesis	Up regulated
Lipid catabolism	Down regulated
Lipid biosynthesis	Down regulated
Oxidative phosphorylation	Down regulated
TCA cycle	Down regulated

As expected the highest increase in expression level occurred for the UCP1 gene, an 8 fold increase in the adipocytes expressing UCP1 when compared to control adipocytes. Moreover, the data revealed that all the genes

encoding for enzymes within the glycolytic, TCA cycle, fatty acid oxidative, oxidative phosphorylation, and mitochondrial biogenesis pathways had decreased expression in UCP1 adipocytes as compared to control adipocytes. For example, hexokinase: the first reaction of glycolysis, its expression level was 22% less in UCP1 expressing cells than control. Phosphofructokinase, the first committed step in glycolysis, its expression level was 28% less. Citrate Synthase, the first step in the TCA cycle, is expression was down 29 % in UCP1 expressing cells as compared to control. Also, NADH dehydrogenase, complex I of the electron transport chain, had its expression level decreased by 20% when comparing the two cell types.

CHAPTER V

CONCLUSIONS

Examination of the data reveals all of the major metabolic pathways to be down regulated with the expression of UCP1 within the cell. One possible explanation for this could be that because of the uncoupling of the oxidative phosphorylation pathway, the cell enters a state of ATP depletion and shuts down the mechanisms necessary for transcription. Furthermore, the review of the expression levels of ubiqutin: an enzyme, whose function is to degrade other proteins, shows a decrease as well. This down regulation would in turn lead to having the cellular enzymes including the ones involved in metabolism function for longer periods of time within the cell. Therefore, the cell can still maintain necessary functions without having to make more of the enzymes.

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APPENDIX

Glycolysis		
	T-test	**UCP1/control**
Hexokinase	0.032226	0.780519536
phosphofructokinase	0.027045	0.724710434
fructobisphosphate aldolase	0.003124	0.546672248
triosephosphate isomerase	0.048193	0.38862309
Glyceraldehyde 3 phosphate dehydrogenase	0.025625	0.487525133
phosphoglycerate kinase	0.013619	0.500681065
pyruvate kinase	0.001477	1.183126047
Glycerogenesis		
PEP carboxykinase	0.018451	1.297985551
glucose 6 phosphatase	0.002062	1.725366205
Glycogen Metabolism		
glycogen phosphorylase	0.012372	0.688879579
glycogen synthase	0.001255	0.540651739
branching enzyme	0.010224	0.356628968
phosphoglucomutase	0.041361	0.759299316
Citric acid cycle		
citrate synthase	0.042259	0.716523987
isocitrate dehydrogenase	0.00989	0.723318154
succinate dehydrogenase	0.040454	0.793064378
fumarase	0.029361	0.716030736
malate dehydrogenase	0.044173	0.788311967
pyruvate dehydrogenase	0.034541	0.805219592
Oxidative Phosphorylation		
ATP synthase	0.032525	0.531069806
ATP synthase	0.019455	0.711261065

ATP synthase	0.011162	0.745276908
ATP synthase	0.042624	0.759738713
NADH dehydrogenase	0.009978	0.736400743
NADH dehydrogenase	0.028682	0.801751834
NADH dehydrogenase	0.013745	0.725320154
NADH dehydrogenase	0.006624	0.735908631
NADH dehydrogenase	0.009424	0.798949461
NADH dehydrogenase	0.020777	0.72122224
succinate dehydrogenase	0.040454	0.793064378
coenzyme Q-cytochrome c oxidoreductase	0.035466	0.735586389
coenzyme Q-cytochrome c oxidoreductase	0.015216	0.739190483
cytochrome c oxidase	0.00014	0.348234763
cytochrome c oxidase	0.022744	0.710150452
Aminoacid Metabolism		
asparagine synthetase	0.009692	0.771154816
Lipid Catabolism		
Carnitine acytransferase II	0.005432	0.635574565
Acyl coa dehydrogenase	0.026676	0.691482326
enoyl coa hydratase	0.000924	0.797711393
beta hydroxyacyl dehydrogenase	0.033687	0.68291956
thiolase	0.03709	0.7940302
propinoyl coa carboxylase	0.012611	0.684424659
Lipid Biosynthesis		
fatty acid synthase	0.023002	0.831595905
Uncoupling Proteins		
uncoupling protein 1	0.017496	8.056634747
uncoupling protein 3	0.027442	0.718022479

Cytochrome P40		
Cytochrome P40	0.027379	0.712114297
Cytochrome P40	0.033757	4.89964546
Cytochrome P40	0.000184	1.562875437
Cytochrome P40	0.03457	1.536311498
Cytochrome P40	0.011692	3.619635454
Cytochrome P40	0.032724	0.787030393
Cytochrome P40	0.033825	2.256590601
Cytochrome P40	0.009485	1.901329882
Cytochrome P40	0.000431	0.63301888

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