

2015

D-Galactose Decreases MitoNEET Levels in Liver Cancer Cells: Impact on Cellular Bioenergetics

Sudip Paudel

Eastern Illinois University

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D-GALACTOSE DECREASES MITONEET LEVELS IN LIVER CANCER

CELLS: IMPACT ON CELLULAR BIOENERGETICS

(TITLE)

BY

SUDIP PAUDEL

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

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**D-GALACTOSE DECREASES MITONEET LEVELS IN LIVER CANCER
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By

Sudip Paudel

Biological Sciences, Eastern Illinois University



A Thesis

Submitted for the Requirements for the Degree of

Master of Science

Under the Direction of

Dr. Michael A. Menze

Department of Biological Sciences

Eastern Illinois University

June 2015

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ABSTRACT

Galactose is a simple sugar that at supraphysiological concentrations accelerates aging and age-related complications, which lead to impaired mitochondrial functions. MitoNEET is a small mitochondrial membrane protein with a molecular mass of 12.2 kDa that functions in diabetes, iron metabolism, regulation of oxidative phosphorylation (OXPHOS) and reactive oxygen species (ROS) homeostasis. MitoNEET knockdown increases cellular respiration rates and ROS levels similar to galactose treatment. Pioglitazone, an antidiabetic drug, binds to mitoNEET and ameliorates galactose toxicity. Cellular mitoNEET levels, exposure of cells to galactose medium, and pioglitazone treatment directly influence cellular respiration. To elucidate the role of mitoNEET in galactose induced toxicity (aging), we measured cellular mitoNEET levels using immunoblotting technique in galactose and pioglitazone treated human liver cancer cells (HepG2), as well as isolated synaptosomes from old and young mice. We also monitored liver cancer cell bioenergetics, using respirometry and calorimetry, of galactose and pioglitazone treated cells. Immunoblotting revealed treatment with galactose reduced endogenous mitoNEET levels and those of a chimeric mitoNEET protein fused to a green fluorescent protein (mNT-GFP), under control of the human cytomegalovirus (CMV) promotor. Furthermore, to determine whether mitoNEET mediates pioglitazone ameliorated galactose toxicity, cells were treated with pioglitazone. Pioglitazone rescued galactose reduced mitoNEET levels in a dose dependent manner. A concentration of 60 μ M pioglitazone reduced cellular mitoNEET levels compared to controls but not mNT-GFP levels. However, pioglitazone at lower concentrations partially restored mitoNEET levels observed with galactose treatment. Since the synthesis of endogenous mitoNEET

and mNT-GFP are regulated by different promoters, D-galactose treatment likely increased degradation rates of mitoNEET. Moreover, unlike galactose treatment in HepG2 cells, immunoblotting revealed higher mitoNEET levels in synaptosomes isolated from aged mice compared to their younger counterparts. We hypothesized that galactose and pioglitazone interact with mitoNEET and alter cellular bioenergetics. Acute treatment with pioglitazone significantly reduced complex I respiration in HepG2 and HepG2-mNT-GFP cells independent of treatment with galactose. Surprisingly, galactose treated HepG2-mNT-GFP cells were less sensitive to pioglitazone treatment. HepG2 cells treated with galactose and HepG2-mNT-GFP cells independent of treatment showed higher OXPHOS activity compared to controls in absence of galactose, but HepG2-mNT-GFP cells did not respond to galactose treatment. Unlike respiration, galactose treatment significantly reduced cellular heat flow in HepG2 and HepG2-mNT-GFP cells measured via calorimetry. However, HepG2-mNT-GFP and pioglitazone treated cells showed higher heat dissipation compared to control HepG2 cells without galactose. Our results show that mitoNEET overexpression increases respiration rates and overall energy transduction in HepG2 cells but does not impact the response to pioglitazone treatment.

DEDICATED TO

To my family for their unconditional love, care and support

ACKNOWLEDGEMENTS

I take the opportunity to thank my advisor, Dr. Michael A. Menze who has been supportive all the time to develop scientific skills during these past two years. Dr. Menze is one of the smartest people I know, the best role model for a scientist and mentor. I hope that I could be as lively, enthusiastic and energetic as Dr. Menze. He has given me the freedom to pursue various projects without objections and has provided insightful and constructional discussion about the research. No words can thank him enough. I would like to give a special thanks to my research committee members Dr. Gary A. Bulla and Dr. Mary E. Konkle for their excellent guidance throughout the years. I am also thankful to Dr. Britto P. Nathan, Dr. Jeffrey R. Laursen, Dr. Karen F. Gaines, Marschelle Y. McCoy (Office Manager) and Sandra P. Baumgartner, (Stockroom Manager) for their commitment to help us succeed in our academic endeavors. I never can forget to appreciate all the support that I received from College of Sciences, Graduate School and Office of International Student and Scholars at Eastern Illinois University to make EIU home away from home for me. I am thankful to Eric Hendricks, Trisha Baily and the undergraduate students in Menze's lab for becoming like my brothers and sisters. My heart-felt thanks and great appreciation goes to the members of Sorptimist International including Jeanne Baxter and Norma Webster for their continuous support. Similarly, great appreciation goes to all Nepalese in Charleston including Dr. Mukti Upadhyay, Sarita Upadhyay, Yagya Rijal, Sunil Thapa, Vaskar Nepal, Manisha Pant, Pabitra Aryal, Sailesh Adhikari and friends. Life would not be as easy without them. Finally, I am indebted for the lifelong and unconditional love, care and support in every step from my parents and family members in California and in Nepal.

TABLE OF CONTENTS

TITLE	i
COPYRIGHT PAGE	ii
ABSTRACT.....	iii
DEDICATED TO	v
ACKNOWLEDGEMENTS.....	vi
LIST OF FIGURES	ix
LIST OF TABLES	xi
INTRODUCTION	1
LITERATURE CITED	10
CHAPTER ONE	
D-GALACTOSE DECREASES MITONEET (CISD1) LEVELS IN HEPG2 CELLS...	17
ABSTRACT.....	17
INTRODUCTION	19
METHODS	22
RESULTS	27
DISCUSSION	33
LITERATURE CITED	35
CHAPTER TWO	
THE EFFECTS OF PIOGLITAZONE ON LIVER CELL BIOENERGETICS	39

ABSTRACT.....	39
INTRODUCTION	41
METHODS	46
RESULTS	48
DISCUSSION	56
LITERATURE CITED	59
CHAPTER THREE	
CO-IMMUNOPRECIPITATION ASSAY IDENTIFIES BINDING PARTNERS OF	
MITONEET	63
ABSTRACT.....	63
INTRODUCTION	64
METHODS	66
RESULTS	68
DISCUSSION	70
LITERATURE CITED	73

LIST OF FIGURES

Chapter 1		Page No
Figure 1	Structure of mitochondrion.	2
Figure 2	Types of redox active iron-sulfur [2Fe-2S] cluster and mitoNEET crystal in a dimer.	2
Figure 3	Advance glycation end product (AGE) formation with galactose, an overview	21
Figure 4	D-galactose supplemented medium reduced endogenous mitoNEET levels.	28
Figure 5	D-galactose, not glucose, is responsible for altered endogenous mitoNEET levels.	28
Figure 6	D-galactose targets degradation of mitoNEET.	29
Figure 7	D-galactose mediated degradation of mitoNEET increases over time.	30
Figure 8	Pioglitazone reduces mitoNEET levels at high concentrations, but restores protein levels at low concentrations.	31
Figure 9	Old mice express higher mitoNEET levels in brain.	32
Chapter 2		
Figure 10	Oxygen consumption (LEAK, OXPHOS and LEAKo) of permeabilized HepG2 and HepG2-mNT-GFP cells cultured in glucose and galactose in response to acute addition of 60 μ M pioglitazone.	49

Figure 11   Glutamate stimulated oxygen consumption of permeabilized   52
HepG2 and HepG2-mNT-GFP cells cultured in glucose or
galactose.

Figure 12   Oxygen consumption of intact HepG2 and HepG2-mNT-GFP   53
cells in response to acute addition of 60 μ M pioglitazone
during uncoupling of the OXPHOS system.

Chapter 3

Figure 13   SDS-PAGE gel after silver staining.   68

LIST OF TABLES

Chapter 2		Page No
Table 1	Overall heat dissipation after addition of pioglitazone to cells in glucose and galactose medium.	54
Table 2	Statistical analysis of change in cellular heat dissipation after galactose and pioglitazone treatment.	55
Table 3	Pairwise correlation analyses change in cellular heat dissipation after galactose and pioglitazone treatment	55
Chapter 3		
Table 4	List of proteins detected using CO-IP, proteomics analysis and database search.	69

INTRODUCTION

Mitochondria

Aerobic mitochondria in mammalian cells are double membrane bounded organelles. The outer membrane is made up of a phospholipid bilayer which is permeable to molecules less than 5000 Da due to the presence of Voltage-dependent Anion Channel (VDAC) proteins (Lemasters et al., 2006). The nearly impermeable inner membrane is significantly different from the outer membrane in composition because of the presence of cardiolipin, respiratory complexes, and remnants of ancestral bacteria (McBride et al., 2006). The inner membrane is folded into compartmentalized structures called cristae. Five different enzymatic complexes, which take part in the terminal breakdown of food in the presence of oxygen termed electron transport chain (ETC), are embedded in the cristae (Koopman et al., 2013). Three complexes out of five (complex I, complex III and complex IV) of the ETC pump protons into the intermembrane space establishing proton motive force (PMF) across the membrane during the reduction of oxygen to water, and the fifth complex generates ATP (Fig. 1) by using the established PMF (Brown 1992).

In terms of energy requirement, every eukaryotic organism depends on organelles of mitochondrial origin (mitosomes) which may or may not generate ATP (Müller et al., 2012). Most eukaryotic organisms contain mitochondria that produce ATP. Aerobic mitochondria exclusively use oxygen as the terminal electron acceptor in the ETC, but other types of mitochondria, such as anaerobic mitochondria (H_2 -producing mitochondria and hydrogenosomes), use chemical compounds other than oxygen for this purpose (Müller et al., 2012). The energy required to fuel mitochondrial activity is derived from organic biomolecules (food materials) which are broken down into simpler units through

a series of stepwise enzymatic reactions. Glucose (6 carbon sugar) for example is being broken down into pyruvate (3 carbon alpha-keto acid) in the cell cytoplasm (anaerobic glycolysis) (Scrutton and Utter 1968). During this process a part of the chemical energy

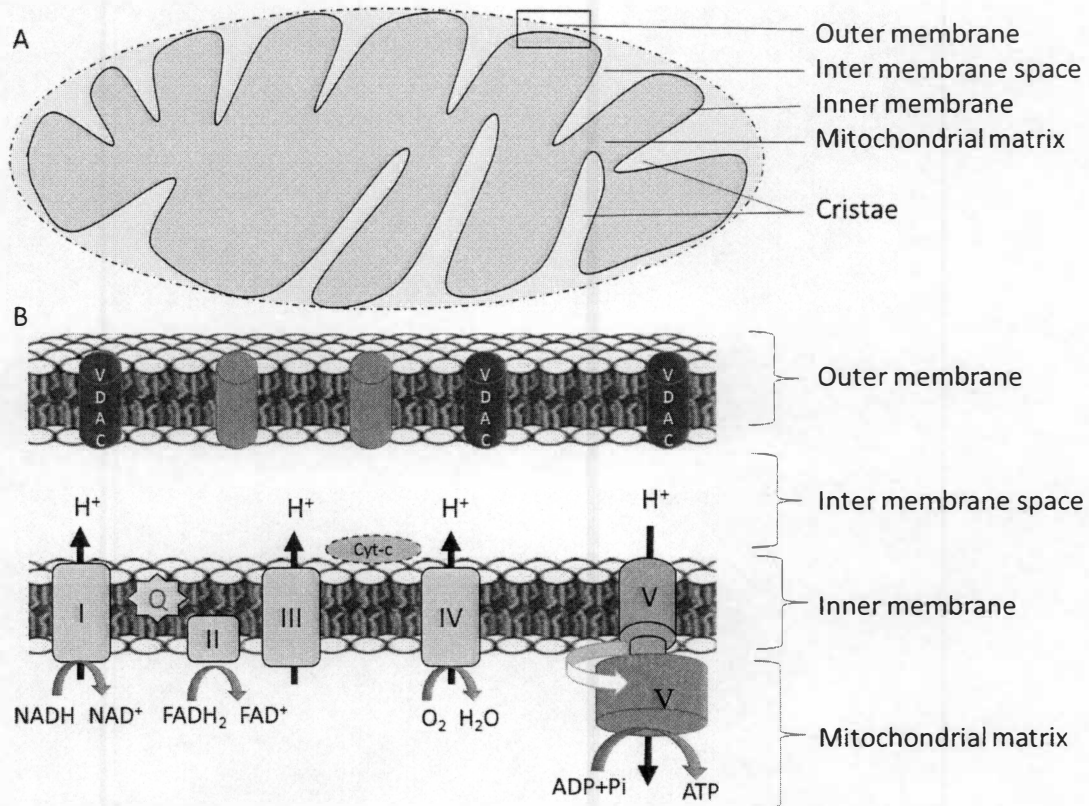


Figure 1. Structure of mitochondrion (A) general outline and (B) in details showing porous outer mitochondrial membrane due to presence of VDAC protein, respiratory complexes (I through V), ubiquinone embedded in inner membrane (Q), cytochrome c in inter membrane space attached to inner membrane (Cyt-c) and flow of proton across the inner membrane (dark arrows), (Eng et al., 2003)

is transferred to the oxidized nicotinamide adenine dinucleotide (NAD⁺) in the form of electrons generating the reduced form of NADH. In canonical oxygen-respiring

mitochondria (rat-liver type mitochondria), pyruvate generated in the cytoplasm is transported to the mitochondria via the mitochondrial pyruvate carrier (Halestrap 1975)

Furthermore, pyruvate is broken down into carbon dioxide (CO_2) and acetyl-Coenzyme A (acetyl-CoA) via pyruvate dehydrogenase complex (Patel and Korotchkina 2006), and acetyl-CoA enters into the tricarboxylic (TCA) acid cycle (also called the Krebs cycle) located within the mitochondrial matrix to generate intra-mitochondrial NADH, FADH_2 (reduced flavin adenine dinucleotide) and CO_2 (Ferne and Carrari 2004). In addition, breakdown of fat takes place in the mitochondrial matrix and peroxisome and both organelles can generate acetyl-CoA (Lazarow 1978, Wallace et al., 2010). Similarly, amino acids are first deaminated and decarboxylated and processed to pyruvate, succinyl-CoA, acetyl-CoA, fumarate, α -keto-glutarate and oxaloacetate, which are intermediate products of the TCA cycle. The intermediates of the TCA cycle are also used for the synthesis of other metabolites and precursors of macromolecules including amino acids, nucleotides etc. (Wallace et al., 2010, Reddy and Reddy 2011). Unlike pyruvate, the cytosolic proton carrier NADH is not transported into the mitochondrial matrix but their oxidation supports the malate-aspartate shuttle system through the cytosolic NADH/cytochrome c electron transport pathway. Two different pathways, the glutamate dehydrogenase pathway and the aspartate aminotransferase pathway, generate α -ketoglutarate inside the mitochondria. The malate-aspartate shuttle system is linked to the α -ketoglutarate generated by glutamate dehydrogenase (Abbrescia et al., 2012).

The mitochondrial matrix is densely packed with enzymes for the Krebs cycle, except succinate dehydrogenase (complex II) which is associated with the inner mitochondrial membrane. Three molecules of reduced NADH, and one molecule of ATP

and FADH₂ are produced from the oxidation of acetyl-CoA and two molecules of CO₂ are released. The electrons from these energy rich molecules are transferred to oxygen through the ETC, which releases energy. During the transfer of electrons from higher energy compounds to lower energy centers, complex I, complex III and complex IV pump protons from the matrix to the intermembrane space generating an electrochemical gradient across the inner membrane (Mitchell 1961).

Mitochondria are semiautonomous cell organelles that contain their own DNA (mtDNA). There are 92 different genes encoding various subunits of the respiratory complexes and ATP synthase proteins in the nuclear DNA and mtDNA. In total, 13 respiratory complexes and ATP synthase proteins subunits, two genes for mitochondrial ribosomal RNAs (mt-rRNA) and 22 mitochondrial transfer RNAs (tRNA) are coded in mtDNA. The remaining proteins that are essential for proper mitochondrial function are encoded by nuclear DNA. For instance, Complex II is exclusively encoded by nuclear DNA (Koopman et al., 2013, Reinecke et al., 2009, Smeitink et al., 2001).

MitoNEET (CISD1)

Background

MitoNEET was discovered as the target protein of anti-diabetic drug pioglitazone (Colca et al., 2004). Localized primarily to the outer membrane of the mitochondria, mitoNEET is an iron-sulfur cluster protein that is postulated regulates cellular metabolic homeostasis such as mitochondrial iron homeostasis, electron transport and oxidative phosphorylation (OXPHOS) (Tamir et al., 2014, Sohn et al., 2013, Wiley et al., 2007). While the exact cellular function(s) of mitoNEET is/are unknown, regulating redox

reactions in the mitochondria, the accumulation of reactive oxygen species, and control of apoptosis are a few of the proposed functions (Geldenhuys et al., 2014). Reduced mitoNEET levels are associated with enhanced mitochondrial iron content, increased respiration, and reduced weight gain in mice on high fat diets. Increased oxidative stress and glucose intolerance are associated with reduced levels of mitoNEET (Kusminski et al., 2012).

Structure of mitoNEET

The protein mitoNEET contains a redox-active iron-sulfur ([2Fe-2S]) cluster in a CDGSH binding domain. The term “mitoNEET” was given based on its close association with the mitochondrion (mito) and its conserved amino acid sequence Asn-Glu-Glu-Thr (NEET) (Colca et al. 2004). MitoNEET is a small protein composed of only 108 amino acid residues with a molecular weight of 12.2 kDa. The theoretical isoelectric point (pI) is 9.20 (ExPASy 2015) MitoNEET contains 12 negatively charged residues (Aspartic acid and Glutamic acid) and 17 positively charged residues (Arginine and Lysine). The computed instability index (II) of 40.55 indicates mitoNEET is an unstable protein, which is also supported by the estimated half-life time of 30 hours (for mammalian reticulocytes), >20 hours (for yeast, *in vivo*) and >10 hours *in vivo* (for *Escherichia coli*, expressed heterologously) (ExPASy 2015). MitoNEET belongs to the CISD (CDGSH iron-sulfur domains) protein family. MitoNEET is attached to the outer mitochondrial membrane by a N-terminal hydrophobic transmembrane helix and the C-terminal hydrophilic CISD domain is facing the cytoplasm (Wiley et al., 2007). The CDGSH domain is a type of zinc finger domain which binds iron rather than zinc as a redox-active

[2Fe-2S] cluster. Recent reports have indicate that Zn^{2+} and $\text{Fe}^{2+/3+}$ compete for the same Zn-finger motif in mitoNEET (Tan et al. 2012).

In humans, there are three CISD proteins present named mitoNEET (CISD1), Miner 1 (CISD2) and Miner2 (CISD3) and they are characterized by presence of one or two CDGSH motifs. CDGSH is a domain of around 40 aa (amino acids 55-93) annotated as a CDGSH-type zinc finger with a motif containing a highly conserved 17 amino acid residue consensus sequence WCXCXX(S/T)XXXPWCDG(S/T/A)H], where W and X are a hydrophobic or any residue, respectively. The function(s) of all CISD proteins are still ill-defined (Wiely et al 2007, Lin et al 2011). The redox-active 2Fe-2S cluster is pH labile and is significantly less stable at pH 4.5 and below when compared to pH 7.0 (Tirrell et al., 2009).

Crystal structures of mitoNEET

The human mitoNEET forms a homodimer in the crystal structure. Each monomer consists of one redox active [2Fe-2S] cluster. Each $\text{Fe}^{2+/3+}$ ion in the clusters is tetrahedral and coordinated with the ligand, a sulfide ion and two amino acid residues, cysteine and histidine. MitoNEET and other NEET proteins contain two domain structures, a cap and a cluster-binding domain (Fig. 2). They are formed by the interlock of each monomer forming a homodimer and their unique folding domain called NEET fold. Both structural features play vital roles in harboring the redox-active 2Fe-2S cluster (Nechushtai et al., 2012). Although other configurations are known, the redox active [2Fe-2S] clusters in CISD proteins might be either ferredoxin or Rieske type configurations. In the ferredoxin configuration, two cysteine sulfur atoms bind to a $\text{Fe}^{2+/3+}$ ion [(Cys)(Cys)FeS2Fe(Cys)(Cys)] (Fig. 2).

The Rieske-type is characterized by two cysteine sulfur, one $\text{Fe}^{2+/3+}$ ion, and the N δ atoms of two histidine ligands bind to the other $\text{Fe}^{2+/3+}$ ion, (His)(His)FeS₂Fe(Cys)(Cys). MitoNEET has unique (Cys)(His)FeS₂Fe(Cys)(Cys) configuration where, as in Rieske centers, N δ nitrogen of the histidine binds to the cluster (Fig. 2) (Dicus et al. 2010).

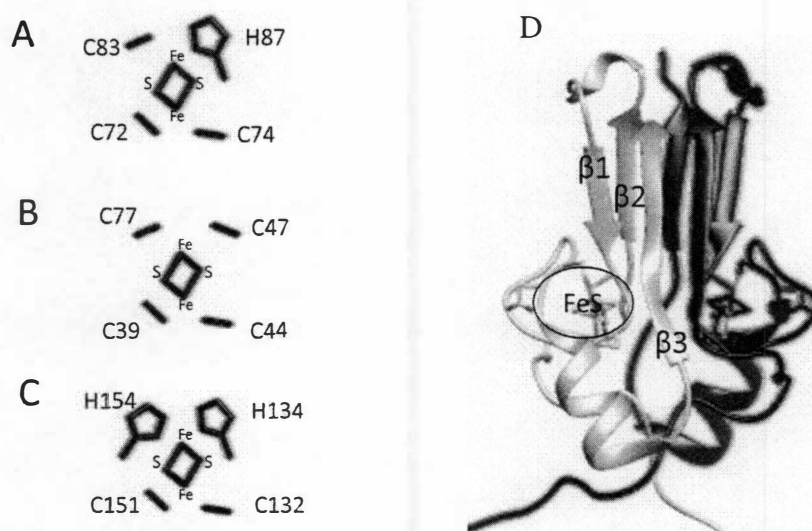


Figure 2. Redox active [2Fe-2S] cluster (Hou et al. 2007). Unlike ferredoxin (B) and rieske (C), mitoNEET has unique 2Fe-2S cluster bound with three Cys residues and one His residue (A), MitoNEET crystal in a dimer (Lin et al. 2011). Each monomer (dark and gray) consists of β - β - α - β (C to N terminal) topology and a Zn-finger loop binding an Iron ion and Sulfur ligand (dark circle). The respective β -sheets of two monomers are anti-parallel to each other (D)

The amino acid residues in the C-terminal part up to Lys-55 form a β - β - α - β topology (Figure 2). However, the N-terminal part from Arg-33 to Lys-55 forms a long loop. The loop between β 2 and α 1 binds a [2Fe-2S] cluster and is flanked by the α -helix and two anti-parallel β -sheets (β 2 and β 3) (Fig. 2). The β 1 region consists of hydrophobic

amino acid residues such as Ile-56, Phe-60, Ala-69, Tyr-71, Phe-80, and Phe-82. The hydrophobic residues interact with the cluster-binding domain (residues from $\beta 2$ to $\beta 3$) (Hou et al. 2007).

A very tight dimerization is created by the multi-hydrophobic interaction between different residues of the two associated monomers. (Hou et al. 2007) The amino acid residues participating in the dimerization are Val-56 and Val-57 from $\beta 1$, Leu-65 from loop between $\beta 1$ and $\beta 2$, Tyr-71 from $\beta 2$, Trp-75 from the loop between $\beta 2$ and $\alpha 1$, Leu-101 and Ile-103 from $\beta 3$. The hydrophobic residues contribute to the dimerization of mitoNEET that covers 30% of the total surface area in each monomer. Around 58% of amino acid residues participate in the dimerization process. During dimerization, the $\beta 1$ strand of the first monomer, residing in the N-terminal end, forms a parallel β -sheet with the $\beta 3$ strand of second monomer resulting in a “face to face” arrangement of two symmetric 3-stranded beta-sheets (Hou et al. 2007).

Crystal structure of related proteins

The protein At5g51720 (*Arabidopsis thaliana*) when truncated from amino acid 39 to 108 has biochemical and biophysical properties comparable with human mitoNEET such as color, absorption peaks in the visible range (at 458 and 530 nm), shift in peaks after reduction (at 440 and 550 nm), etc.. The high level of sequence similarity and various identical biochemical and biophysical properties of At5g51720 protein demonstrate the occurrence of NEET proteins in plants (Nechushtai et al. 2012). In addition, At5g51720 contains the mitoNEET type of 2Fe-2S cluster, coordinated by a (Cys)(His)FeS₂Fe(Cys)₂ ligand, in the CDGSH domain. However, unlike the N-terminal transit sequence in human mitoNEET, At5g51720 contains a cleavable N-terminal leader

sequence for the stroma of chloroplast and the mitochondria. The peptide cleavage site of At5g51720 transit for chloroplast is V-[R/K]↓A-E (Su et al. 2013).

Thesis Objective

Recent studies show that mitoNEET plays an important role in mitochondrial respiration, but little is known regarding the impact of different levels of mitoNEET on cellular respiration, cell proliferation rate and cellular energy transduction. The purpose of this study was to elucidate probable role of mitoNEET in liver cancer cells (HepG2 cells) and its ligand pioglitazone on galactose-induced alteration in cellular bioenergetics.

LITERATURE CITED

- Abbrescia, D. I., La Piana, G., & Lofrumento, N. E. (2012). Malate–aspartate shuttle and exogenous NADH/cytochrome c electron transport pathway as two independent cytosolic reducing equivalent transfer systems. *Archives of Biochemistry and Biophysics*, 518(2), 157-163.
- Borcar, A., Menze, M. A., Toner, M., & Hand, S. C. (2013). Metabolic preconditioning of mammalian cells: mimetic agents for hypoxia lack fidelity in promoting phosphorylation of pyruvate dehydrogenase. *Cell and Tissue Research*, 351(1), 99-106.
- Brown, G. C. (1992). Control of respiration and ATP synthesis in mammalian mitochondria and cells. *Biochem. J*, 284, 1-13.
- Brunmair, B., Staniek, K., Gras, F., Scharf, N., Althaym, A., Clara, R., ... & Fürnsinn, C. (2004). Thiazolidinediones, Like Metformin, Inhibit Respiratory Complex IA Common Mechanism Contributing to Their Antidiabetic Actions?. *Diabetes*, 53(4), 1052-1059.
- Colca, J. R., McDonald, W. G., Waldon, D. J., Leone, J. W., Lull, J. M., Bannow, C. A., Lund, E. T. & Mathews, W. R. (2004). Identification of a novel mitochondrial protein (“mitoNEET”) cross-linked specifically by a thiazolidinedione photoprobe. *American Journal of Physiology-Endocrinology and Metabolism*, 286(2), E252-E260.
- Dicus, M. M., Conlan, A., Nechushtai, R., Jennings, P. A., Paddock, M. L., Britt, R. D., & Stoll, S. (2010). Binding of histidine in the (Cys) 3 (His) 1-coordinated [2Fe–

- 2S] cluster of human mitoNEET. *Journal of the American Chemical Society*, 132(6), 2037-2049.
- Eng, C., Kiuru, M., Fernandez, M. J., & Aaltonen, L. A. (2003). A role for mitochondrial enzymes in inherited neoplasia and beyond. *Nature Reviews Cancer*, 3(3), 193-202.
- Fernie, A. R., Carrari, F., & Sweetlove, L. J. (2004). Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion in Plant Biology*, 7(3), 254-261.
- García-Ruiz, I., Solís-Muñoz, P., Fernández-Moreira, D., Muñoz-Yagüe, T., & Solís-Herruzo, J. A. (2013). Pioglitazone leads to an inactivation and disassembly of complex I of the mitochondrial respiratory chain. *BMC Biology*, 11(1), 88.
- Geldenhuis, W. J., Leeper, T. C., & Carroll, R. T. (2014). MitoNEET as a novel drug target for mitochondrial dysfunction. *Drug Discovery Today*, 19(10), 1601-1606.
- Halestrap, A. P. (1975). The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. *Biochem. J*, 148, 85-96.
- Hou, X., Liu, R., Ross, S., Smart, E. J., Zhu, H., & Gong, W. (2007). Crystallographic studies of human MitoNEET. *Journal of Biological Chemistry*, 282(46), 33242-33246.
- <http://web.expasy.org/protparam/> retrived on 03/18/2015
- Hunter, R. L., Choi, D. Y., Ross, S. A., & Bing, G. (2008). Protective properties afforded by pioglitazone against intrastriatal LPS in Sprague–Dawley rats. *Neuroscience Letters*, 432(3), 198-201.

- Kaundal, R. K., & Sharma, S. S. (2010). Peroxisome proliferator-activated receptor gamma agonists as neuroprotective agents. *Drug News and Perspectives*, 23(4), 241-256.
- Knouff, C., & Auwerx, J. (2004). Peroxisome proliferator-activated receptor- γ calls for activation in moderation: lessons from genetics and pharmacology. *Endocrine Reviews*, 25(6), 899-918.
- Koopman, W. J., Distelmaier, F., Smeitink, J. A., & Willems, P. H. (2013). OXPHOS mutations and neurodegeneration. *The EMBO Journal*, 32(1), 9-29.
- Kounosu, A., Iwasaki, T., Baba, S., Hayashi-Iwasaki, Y., Oshima, T., & Kumasaka, T. (2008). Crystallization and preliminary X-ray diffraction studies of the prototypal homologue of mitoNEET (Tth-NEET0026) from the extreme thermophile *Thermus thermophilus* HB8. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, 64(12), 1146-1148.
- Kusminski, C. M., Holland, W. L., Sun, K., Park, J., Spurgin, S. B., Lin, Y., Askew, G. R., Simcox, J. A., McClain, D. A., Li, C., & Scherer, P. E. (2012). MitoNEET-driven alterations in adipocyte mitochondrial activity reveal a crucial adaptive process that preserves insulin sensitivity in obesity. *Nature Medicine*, 18(10), 1539-1549.
- Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., & Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). *Journal of Biological Chemistry*, 270(22), 12953-12956.

- Lemasters, J. J., & Holmuhamedov, E. (2006). Voltage-dependent anion channel (VDAC) as mitochondrial governor—thinking outside the box. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1762(2), 181-190.
- Lin, J., Zhang, L., Lai, S., & Ye, K. (2011). Structure and molecular evolution of CDGSH iron-sulfur domains. *PloS One*, 6(9), e24790.
- McBride, H. M., Neuspiel, M., & Wasiak, S. (2006). Mitochondria: more than just a powerhouse. *Current Biology*, 16(14), R551-R560.
- Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*, 191(4784), 144-148.
- Müller, M., Mentel, M., van Hellemond, J. J., Henze, K., Woehle, C., Gould, S. B., ... & Martin, W. F. (2012). Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. *Microbiology and Molecular Biology Reviews*, 76(2), 444-495.
- Nechushtai, R., Conlan, A. R., Harir, Y., Song, L., Yogev, O., Eisenberg-Domovich, Y., Livnah, O., Michaeli, D., Rosen, R., Ma, V., Luo, Y., Zuris, J.A., Paddock, M.L., Cabantchik, Z.I., Jennings, P.A. & Mittler, R. (2012). Characterization of Arabidopsis NEET reveals an ancient role for NEET proteins in iron metabolism. *The Plant Cell Online*, 24(5), 2139-2154.
- Paddock, M. L., Wiley, S. E., Axelrod, H. L., Cohen, A. E., Roy, M., Abresch, E. C., Capraro, D., Murphy, A.N., Nechushtai, R., Dixon, J.E. & Jennings, P. A. (2007). MitoNEET is a uniquely folded 2Fe-2S outer mitochondrial membrane protein stabilized by pioglitazone. *Proceedings of the National Academy of Sciences*, 104(36), 14342-14347.

- Patel, M. S., & Korotchkina, L. G. (2006). Regulation of the pyruvate dehydrogenase complex. *Biochemical Society Transactions*, 34(2), 217-222.
- Prakash, A., & Kumar, A. (2013). Pioglitazone alleviates the mitochondrial apoptotic pathway and mito-oxidative damage in the d-galactose-induced mouse model. *Clinical and Experimental Pharmacology and Physiology*, 40(9), 644-651.
- Rabøl, R., Larsen, S., Højberg, P. M. V., Almdal, T., Boushel, R., Haugaard, S. B., ... & Dela, F. (2010). Regional anatomic differences in skeletal muscle mitochondrial respiration in type 2 diabetes and obesity. *The Journal of Clinical Endocrinology & Metabolism*, 95(2), 857-863.
- Reddy, P. H., & Reddy, T. P. (2011). Mitochondria as a therapeutic target for aging and neurodegenerative diseases. *Current Alzheimer Research*, 8(4), 393.
- Reinecke, F., Smeitink, J. A., & Van Der Westhuizen, F. H. (2009). OXPHOS gene expression and control in mitochondrial disorders. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1792(12), 1113-1121.
- Sanz, M. N., Sánchez-Martín, C., Demaille, D., Vial, G., Rigoulet, M., El-Mir, M. Y., & Rodríguez-Villanueva, G. (2011). Acute mitochondrial actions of glitazones on the liver: a crucial parameter for their antidiabetic properties. *Cellular Physiology and Biochemistry*, 28(5), 899.
- Scrutton, M. C., & Utter, M. F. (1968). The regulation of glycolysis and gluconeogenesis in animal tissues. *Annual review of biochemistry*, 37(1), 249-302.
- Smeitink, J., van den Heuvel, L., & DiMauro, S. (2001). The genetics and pathology of oxidative phosphorylation. *Nature Reviews Genetics*, 2(5), 342-352.

- Sohn, Y. S., Tamir, S., Song, L., Michaeli, D., Matouk, I., Conlan, A. R., ... & Mittler, R. (2013). NAF-1 and mitoNEET are central to human breast cancer proliferation by maintaining mitochondrial homeostasis and promoting tumor growth. *Proceedings of the National Academy of Sciences*, 110(36), 14676-14681.
- Song, X. U., Bao, M., Li, D., & Li, Y. M. (1999). Advanced glycation in D-galactose induced mouse aging model. *Mechanisms of Ageing and Development*, 108(3), 239-251.
- Su, L. W., Chang, S. H., Li, M. Y., Huang, H. Y., Jane, W. N., & Yang, J. Y. (2013). Purification and biochemical characterization of Arabidopsis At-NEET, an ancient iron-sulfur protein, reveals a conserved cleavage motif for subcellular localization. *Plant Science*, 213, 46-54.
- Tait, S. W., & Green, D. R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology*, 11(9), 621-632.
- Tan, G., Landry, A. P., Dai, R., Wang, L., Lu, J., & Ding, H. (2012). Competition of zinc ion for the [2Fe-2S] cluster binding site in the diabetes drug target protein mitoNEET. *Biometals*, 25(6), 1177-1184.
- Tirrell, T. F., Paddock, M. L., Conlan, A. R., Smoll Jr, E. J., Nechushtai, R., Jennings, P. A., & Kim, J. E. (2009). Resonance Raman studies of the (His)(Cys) 3 2Fe-2S cluster of MitoNEET: comparison to the (Cys) 4 mutant and implications of the effects of pH on the labile metal center. *Biochemistry*, 48(22), 4747-4752.

- Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*, 324(5930), 1029-1033.
- Wallace, D. C., Fan, W., & Procaccio, V. (2010). Mitochondrial energetics and therapeutics. *Annual Review of Pathology*, 5, 297.
- Wiley, S. E., Murphy, A. N., Ross, S. A., van der Geer, P., & Dixon, J. E. (2007). MitoNEET is an iron-containing outer mitochondrial membrane protein that regulates oxidative capacity. *Proceedings of the National Academy of Sciences*, 104(13), 5318-5323.
- Willson, T. M., Lambert, M. H., & Kliewer, S. A. (2001). Peroxisome proliferator-activated receptor γ and metabolic disease. *Annual Review of Biochemistry*, 70(1), 341-367.
- Xu, D., Murakoshi, N., Igarashi, M., Hirayama, A., Ito, Y., Seo, Y., Tada, K. & Aonuma, K. (2012). PPAR- γ Activator Pioglitazone Prevents Age-Related Atrial Fibrillation Susceptibility by Improving Antioxidant Capacity and Reducing Apoptosis in a Rat Model. *Journal of Cardiovascular Electrophysiology*, 23(2), 209-217.

CHAPTER ONE

D-GALACTOSE DECREASES MITONEET (CISD1) LEVELS IN HEPG2 CELLS

ABSTRACT

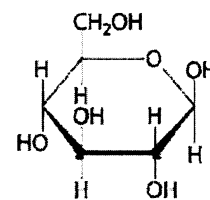
Supra-physiological levels of D-galactose accelerate aging in rodents by increasing the production of reactive oxygen species (ROS). However, the underlying mechanism of galactose action is still poorly resolved. Similar to galactose treatment, mitoNEET deficiency decreases iron content in the mitochondrial matrix and increases mitochondrial ROS production. MitoNEET is a small iron-sulfur cluster containing protein localized to mitochondrial membranes and was discovered as a target protein of the drug pioglitazone. Pioglitazone, an antidiabetic drug, also ameliorates galactose mediated aging processes, such as memory deficits and decreased motor activities in a mouse model of aging. To investigate the role(s) of mitoNEET in galactose-induced aging, we exposed human hepatocellular carcinoma cells (HepG2) to 10 mM D-galactose and measured endogenous mitoNEET levels by immunoblotting. Galactose treatment for 2 weeks significantly reduced mitoNEET levels. This effect was observed both in presence or absence of glucose in the culture medium. D-galactose also decreased mitoNEET tagged with green fluorescent protein (mNT-GFP) levels in stably transfected HepG2 cells under the control of the human cytomegalovirus (CMV) promoter. Furthermore, treatment of HepG2 cells with 60 μ M pioglitazone significantly reduced mitoNEET levels similar to D-galactose but at low concentration increased protein concentrations. Since the synthesis of endogenous mitoNEET and mNT-GFP are regulated by different promoters, D-galactose most likely increases degradation rates of mitoNEET. Immunoblotting revealed higher mitoNEET levels in synaptosomes isolated

from aged mice compared to their younger counterparts. This report highlights the links mitoNEET to a D-galactose induced model of accelerated aging.

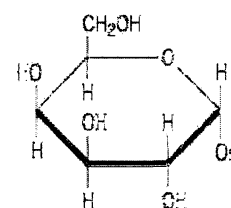
INTRODUCTION

Galactose mediated aging models: Impact of pioglitazone

Peroxisome proliferator-activator receptor gamma (PPAR γ) is a transcription factor which include 48 transcription factors. The activity of PPAR γ is regulated by direct binding of lipid metabolites, hormones such as steroid and thyroid, xenobiotics and vitamins (Kersten et al., 2000). Several target genes that PPAR γ stimulate such as genes encoding lipoprotein lipase, fatty-acid transport protein, oxidized LDL receptor 1 play a role in metabolic homeostasis including increased insulin sensitization and lipid circulation thereby lowering glucose, differentiation and maturation of adipocytes. Likewise, PPAR γ stimulation is associated with increased food intake in brain and increased antigen uptake in dendritic cells (Brunmair et al, 2004, Knouff and Auwerx 2004, Willson et al., 2001, Lehmann et al., 1995 and Wiley et al, 2007).

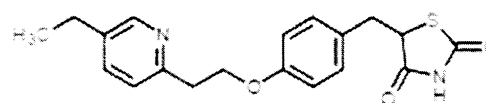


D-glucose



D-galactose

The PPAR γ agonist thiazolidinedione (TZD) family members, pioglitazone for example, induce the PPAR γ coactivator 1-alpha (PGC-1 α) (Hondares et al., 2006). Transcriptional co-activators PGC-1 α , PGC-1 β and PGC-1-related co-activator (PRC) are the members of PPAR- γ coactivators-1 (PGC-1). These co-activators have a vital role in



Pioglitazone

maintaining cellular metabolic activities including carbohydrate and lipid metabolism, reactive oxygen species homeostasis and energy homeostasis. Furthermore, the

antioxidant proteins glutathione-peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) also belong to the PGC-1 family (Salem et al., 2012, Hondares et al., 2006). Pioglitazone, a glucose lowering drug in type II diabetic patients, has been found to bind mitoNEET (Colca et al., 2004) as well as reduce mitochondrial activity by inhibiting complex I activity in liver tissue and skeletal muscle tissue (García-Ruiz et al., 2013, Sanz et al., 2011, Tait and Green 2010, Rabøl et al., 2010).

Aging is characterized by reduced mitochondrial function, stress intolerance, decline of anatomical integrity and function of tissue and organ systems. Although, the mechanism of these processes is still unknown, genetic and environmental factors trigger aging (Semba et al., 2010, Sahin 2010). According to the metabolism theory, aging is the consequential phenotype of a metabolism disorder of the body (Tang and He 2013). Similar to biological aging and various age-related neurodegenerative diseases, long term exposure of D-galactose induces progressive mitochondrial dysfunctions, and to a decline in learning and memory capacity in a rodent model (Lei et al. 2008, Song et al., 1999) but the mechanism of this toxicity is unknown.

A reducing sugar such as D-galactose elevates advanced glycation end-products (AGEs) in animal cells and tissues (Schalkwijk et al., 2004, Semba et al., 2010, Singh et al., 2001, Song et al., 1999). The carbonyl group of galactose reacts with primary amino groups of proteins, and due to glycooxidation and auto-oxidation, highly reactive carbonyl compounds such as carboxymethyl-lysine and hydroimidazolone are formed. These reactive carbonyl compounds react with lipids, nucleic acids and proteins forming a group of heterogeneous macromolecules, the AGEs (Schalkwijk et al., 2004, Singh et al., 2001). An elevation of AGEs levels in cells is responsible for a significant glucose

metabolism disorder, increase in reactive oxygen species (ROS) and thereby causing abnormal metabolism of tissues and organs including heart, liver, kidney, brain and other important organs. Furthermore, galactose is reduced into galactitol and increases lipid peroxidation, damage cell membranes leading to an acceleration of aging (Semba et al., 2010, Song et al., 1999). Therefore, galactose has been used as a tool to study accelerated aging, oxidative stress, and tissue damage in animal models (Tang and He 2013, Parameshwaran et al., 2010, Song et al., 1999).

Although the mechanism is unknown, the consequence of binding of pioglitazone to mitoNEET along with PPAR γ stimulation plays a protective role against galactose induced aging in a rodent model (Prakash and Kumar

2013, Hunter et al., 2008). To investigate the involvement of mitoNEET in aging processes, we measured mitoNEET levels in HepG2 cells exposed to galactose and in synaptosomes isolated from young and old mice brains.

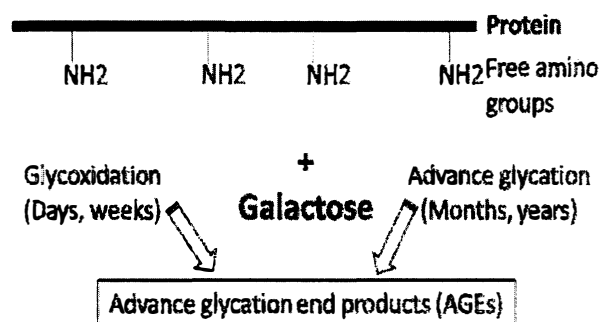


Figure 3. Advance glycation end product (AGE) formation with galactose, an overview

METHODS

Chemicals

All chemicals for respiration measurements were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). Water for solution preparation was purified with a Milli-Q Reagent Water System (Billerica, MA) to an electrical resistance of 18 mΩ.

Cell Culture

Human hepatocellular carcinoma cells (HepG2) were obtained from the American Type Culture Collection (Manassas, VA) and grown in 75 cm² cell culture flasks (Corning Incorporated, Corning, NY). Standard cell culture medium was composed of Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA) supplemented with 5.5% fetal bovine serum (FBS) purchased from Atlanta Biologicals Inc. (Flowery Branch, GA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (MP Biomedicals, Santa Anna, CA). Treatment groups were switched to DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10 mM D-galactose, 15 mM glucose, and both 10 mM D-galactose and 15 mM galactose at least two weeks prior to experimentation. Dialyzed FBS was used for 0 mM glucose treatment. The cells were maintained in a humidified atmosphere of 6.5% CO₂ and 93.5% air at 37 °C and the culture medium was renewed every 3-4 days. The cells were sub-cultured every seven days or before reaching ~90% confluency. To sub-culture, cells were dissociated using 0.25% trypsin plus 1 mM EDTA in balanced salt solution (Invitrogen, Carlsbad, CA) and reseeded at 1.5×10^6 cells per 75 cm² cell culture flasks.

Cloning, transfections and generation of stable cell lines

pCMV6-AC-GFP (RC203308L1) (OriGene Technologies, Inc, Rockville, MD 20850) vector was used to generate green fluorescent protein tagged mitoNEET cell lines (HepG2-mNT-GFP). The protocol for transfection of HepG2 cells grown in 2500 μ L of medium in 35 mm dishes using a FuGENE®HD:DNA ratio of 4.5:1 was followed to generate the cell line. Briefly, HepG2 cells were plated a day prior to transfection at a density of 80% per well in a 35 mm dish in 2 ml of complete growth medium (DMEM + 10% FBS). DNA solution at the concentration of 2.5 μ g per well was added after mixing DNA and reagent (FuGENE®HD) at the ratio of 4.5:1 in 125 μ L of medium. The transfected cells were selected using neomycin (G418).

Impact of galactose and glucose on mitoNEET levels

SDS-PAGE and Western blotting techniques were used to study the impact of galactose and glucose on mitoNEET levels. Proteins isolated from HepG2 cells and HepG2-mNT-GFP cells cultured in DMEM supplemented with D-galactose, D-galactose with dialyzed FBS, glucose and both galactose and glucose were used. Proteins were isolated from $\sim 5 \times 10^6$ cells of the respective treatment groups using RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) following the manufacturer's protocol (Thermoscientific # 89900) and the protein concentration was determined using Thermo Scientific, Pierce™ Coomassie (Bradford) Protein Assay Kit according to the instructions of the manufacturer (Thermo Fisher Scientific, Waltham, MA).

Synaptosome Isolation from mouse brain

Brain synaptosomes from young (3-6 months) and old (12+ months) female WT (C57BL/6J) littermate mice were used in this study. Synaptosomes were isolated by Eric Hendricks for his thesis work as described by Kristian (2010) with minor modification and kindly provided to us (Hendricks 2015, Master's Thesis). Briefly, the forebrain was removed, washed, and homogenized in 20 mL of isolation medium (IM) containing sucrose (225 mM), mannitol (75 mM), EGTA (1 mM), HEPES (5 mM), and 1 g/L BSA adjusted to pH 7.4 with Tris base. The homogenate was transferred evenly into two 10 mL polycarbonate tubes and centrifuged at 1300g for 3 min. The pellet was resuspended in 3 mL of IM and centrifuged at 1300g for an additional 3 min. The supernatants from the two centrifugations were combined and centrifuged at 21,000g for 10 min. A Percoll gradient in IM was prepared in a separate polycarbonate tube by first adding 3.7 mL of 24% Percoll and, using a transfer pipette, 1.5 mL of 40% Percoll was slowly added underneath the 24% Percoll layer. The supernatant from the previous centrifugation step was discarded and the pellet was re-suspended in 3.5 mL of 15% Percoll, and the re-suspended pellet was next layered on top of the 24/40% Percoll gradient using a transfer pipette, and then centrifuged at 30,700g for 8 min. The material at the 15% Percoll band, containing mostly myelin, was discarded, whereas the 24% Percoll band, containing mostly synaptic mitochondria, was collected. The fraction containing the synaptosomes was washed with 6 mL of IM and centrifuged at 16,700g for 10 min. This step was repeated and the supernatant was discarded both times. The resulting pellet was re-suspended in 400 μ L of IM and contained 6-7mg/mL synaptosomal protein, which was

determined using a Percoll friendly Modified Lowry Protein Assay Kit according to the instructions of the manufacturer (Thermo Fisher Scientific, Waltham, MA).

SDS-PAGE and Western blot analyses.

Isolated proteins were subjected to 12% SDS-PAGE using a Bio-Rad Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Hercules, CA). Proteins were denatured at 96°C for 5 min in sample buffer (2% SDS, 25% glycerol, 5% β -mercaptoethanol, 0.01% bromphenol blue, and 62.5 mM Tris-HCl, pH 6.8), and 15–20 μ g of protein were loaded per lane. After electrophoresis, proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane (0.2 mm, Bio-Rad) in a transfer buffer (192 mM glycine, 20% methanol, 0.025% SDS, and 25 mM Tris) by use of a Bio-Rad Mini Trans-Blot. Membranes were stained with Ponceau S (Sigma-Aldrich 0.1% v/v glacial acetic acid, 0.011% w/v Ponceau S) to confirm transfer of the proteins. The nitrocellulose membrane was blocked using blocking buffer (5% w/v milk powder in TBS-T, 20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.1% Tween-20) for 1 hour. Anti-CISD1 [2B3] mouse monoclonal antibody (ab118027), rabbit polyclonal antibodies anti-VDAC antibody (Cell Signaling, #D7312), anti- β -tubulin (Cell Signaling, #2128S) and anti- β -actin antibody (Cell Signaling, #4967S) were used as primary antibodies at 1:5,000 dilutions. The blots were incubated overnight with the primary antibody in blocking buffer at 4°C. Biotinylated goat anti-rabbit IgG and goat anti-mouse IgG (Vector Laboratories) were used as secondary antibodies at dilutions of 1:5,000. Blots were stained and developed using Amersham Hyperfilm^{TM} (GE healthcare Ltd, #28906837) and LumiGLO (Cell Signaling Technology, Danvers, MA #7003), following the protocol of the manufacturer.

Western Blotting band analysis

ImageJ (imagej.nih.gov/ij/ National Institutes of Health) open access software was used to analyze the band intensities. The bands intensities were expressed in terms of percentage of a cellular housekeeper and/or a mitochondrial housekeeper protein (β -tubulin, β -actin and/or VDAC).

Statistical Analysis

Student t-test using Microsoft excel was used to analyze Western blot data. A stepwise multiple comparisons procedure (Newman–Keuls method) was used to identify sample means that are significantly different from each other.

RESULTS

D-galactose decreased cellular mitoNEET levels in HepG2 cells

HepG2 cells were grown in DMEM supplemented with 10 mM galactose for two weeks. The two proteins β -actin and VDAC were chosen as cellular and mitochondrial housekeeper proteins, respectively. Western blotting showed galactose treatment significantly reduced mitoNEET levels to 29.4 ± 2.16 % of β actin from 42.73 ± 4.28 % of β actin in high glucose medium (Fig. 4). To investigate whether galactose or glucose is responsible for the altered levels of mitoNEET, we used a low glucose medium and supplemented with 15 mM glucose alone, 10 mM galactose alone or the combination of 15 mM glucose plus 10 mM galactose. Western blotting showed galactose decreased the mitoNEET levels irrespective of presence of the glucose and band intensities of 45.8 ± 5.35 % of controls were found in samples cultured in 10 mM galactose alone, 72.6 ± 4.37 % of controls in 15 mM glucose and 10 mM galactose together (Fig. 5). No changes in the amount of VDAC or β -actin (Fig. 4 and Fig. 5) were found among the treatment groups verifying that both proteins are suited to serve as housekeeper proteins. Furthermore, the results support the interpretation that mitoNEET levels per mitochondrion were decreased due to galactose treatment and not as a result of reductions in mitochondrial quantity per cell.

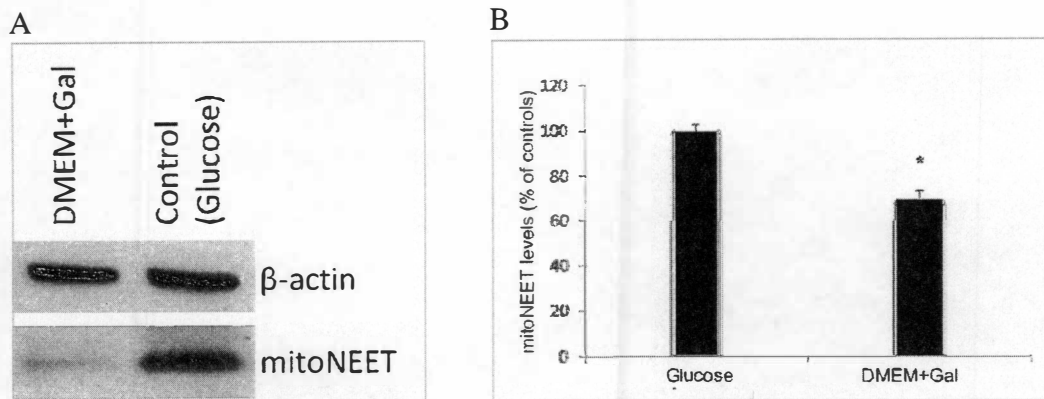


Figure 4. Galactose supplemented medium reduced endogenous mitoNEET levels. (A) Western blot showing mitoNEET levels in HepG2 cells cultured in 10 mM galactose supplemented medium (DMEM + Gal), and high glucose containing medium (control) (B) Band intensities were quantified using ImageJ software ($p < 0.05$, $n = 4$, $\pm$ SEM). * statistically significant differences between different treatment groups.

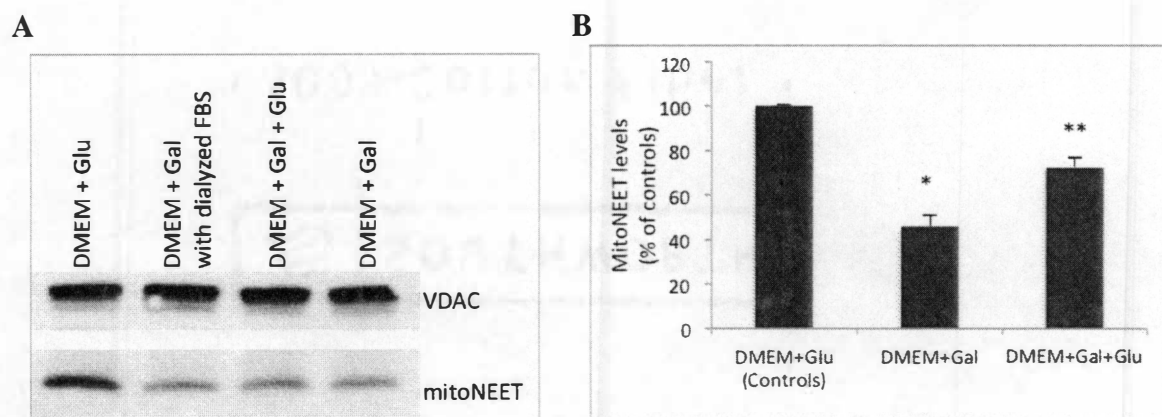


Figure 5: Galactose, not glucose, is responsible for altered endogenous mitoNEET levels. (A) Western blot showing mitoNEET levels in HepG2 cells cultured in 10 mM galactose (gal), 15 mM glucose (glu) and in presence of both galactose and glucose (gal/glu). (B) Band intensities were quantified using ImageJ software ($p < 0.05$, $n = 3$, $\pm$ SEM). (*) indicate statistically significant differences between different treatment groups.

D-galactose likely targets degradation of cellular mitoNEET in HepG2 cells

The synthesis of cellular mitoNEET is endogenously regulated by *cisd1* gene located on chromosome 10 (Tamir et al., 2015, <http://www.ncbi.nlm.nih.gov/gene/55847>) HepG2 cells were stably transfected with a chimeric protein composed of green fluorescent protein (GFP) attached to the C-terminus of mitoNEET (mNT-GFP). Transcription of the construct was under the control of the human cytomegalovirus virus (CMV) promotor. After stable clones were obtained, the mNT-GFP cells were cultured in low glucose medium supplemented with 10 mM D-galactose for two weeks. Similar to the levels of endogenous mitoNEET in control HepG2 cells (Fig. 5), the galactose treatment significantly reduced the endogenous mitoNEET levels to 75.2 ± 2.4 % of controls in GFP-mNT-GFP cells. Surprisingly, not only were the endogenous mitoNEET

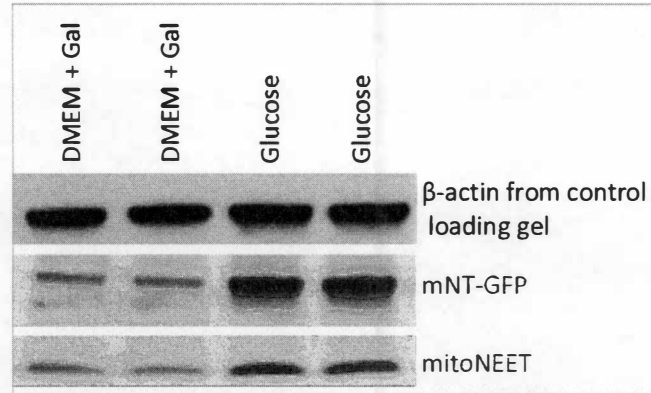


Figure 6: Galactose targets degradation of mitoNEET. Western blot showing endogenous mitoNEET, as well as green fluorescent protein tagged mitoNEET (mNT-GFP) levels in HepG2-mNT-GFP cells cultured in galactose or glucose supplemented medium. Galactose reduced both endogenous as well as mNT-GFP. Band intensities were quantified using ImageJ software ($p < 0.05$, $n = 3$, $\pm$ SEM).

levels reduced, but galactose also lowered the levels the chimeric protein mNT-GFP to 52.7 ± 9.39 % of controls (Fig. 6), suggesting that galactose increases degradation rates of mitoNEET.

Time course of mitoNEET degradation

HepG2 cells maintained in high glucose medium were transferred to the medium supplemented with 10 mM D-galactose and the levels of endogenous mitoNEET were

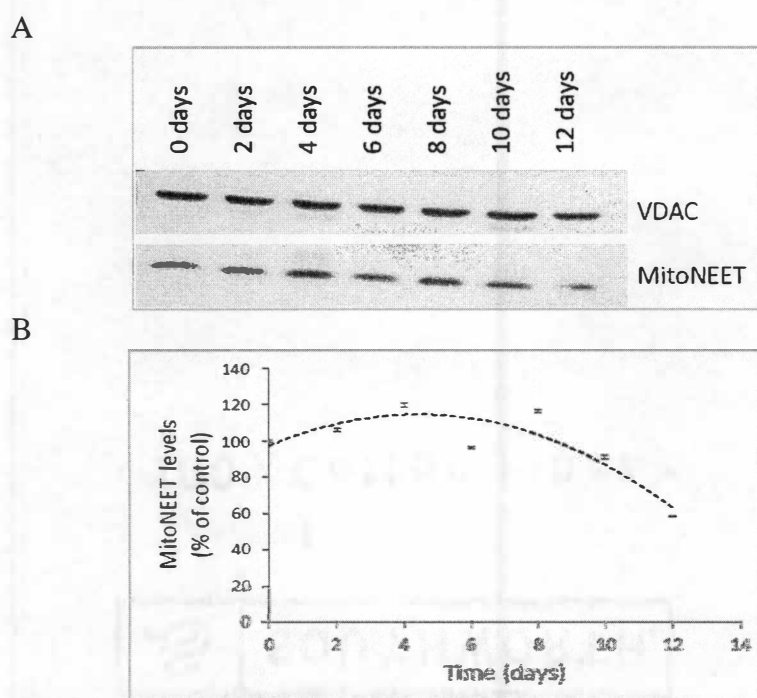


Figure 7: D-galactose mediated degradation of mitoNEET increases over time. (A) Western blot showing mitoNEET levels in HepG2 cells after switching the medium glucose to galactose (B) Band intensities were quantified using ImageJ software ($p < 0.05$, mean $\pm$ SEM), curve is only for the illustration purpose.

measured every other day for two weeks. Western blotting shows galactose significantly reduced endogenous mitoNEET levels in two weeks compared to the controls (Fig. 7).

Pioglitazone at high concentration reduces but at low concentration partially restores mitoNEET

HepG2 cells cultured in high glucose medium or in presence of 10 mM galactose supplemented medium were treated with different concentration of pioglitazone for 24 h. Again, β actin and VDAC were used as cellular housekeeper and mitochondrial

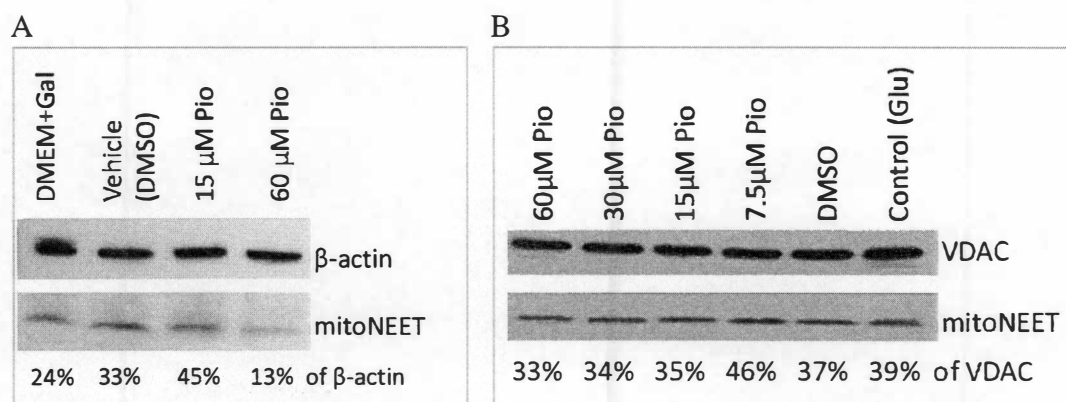


Figure 8. Pioglitazone reduces mitoNEET levels at high concentrations, but restores protein levels at low concentrations. Western blot showing mitoNEET levels in HepG2 cells treated with different concentration of pioglitazone (pio) cultured in (A) 10 mM galactose and (B) high glucose medium. Band intensities were quantified using ImageJ software.

housekeeper proteins, respectively. Western blotting revealed pioglitazone at a concentration of 60 μ M significantly reduced endogenous mitoNEET levels similar to D-galactose but at 7.5 μ M and 15 μ M partially restored endogenous mitoNEET levels in both high glucose and galactose supplemented mediums (Fig. 8).

MitoNEET expression is elevated in the synaptosomes of aged mice

Synaptosomes (mitochondria located in neuronal cell terminals) were isolated from the forebrain of young (3-4 month old) and old (more than 12 months old) mice.

The endogenous mitoNEET levels were measured using the Western blotting technique. Two proteins which are synthesized regularly in the cells (β -actin and β -tubulin) were used as control for cellular housekeeping proteins and the outer mitochondrial protein VDAC, served as control for a mitochondrial housekeeping protein. Western blotting showed significant increases in endogenous mitoNEET levels in aged mice compared to the young counterparts (Fig. 9).

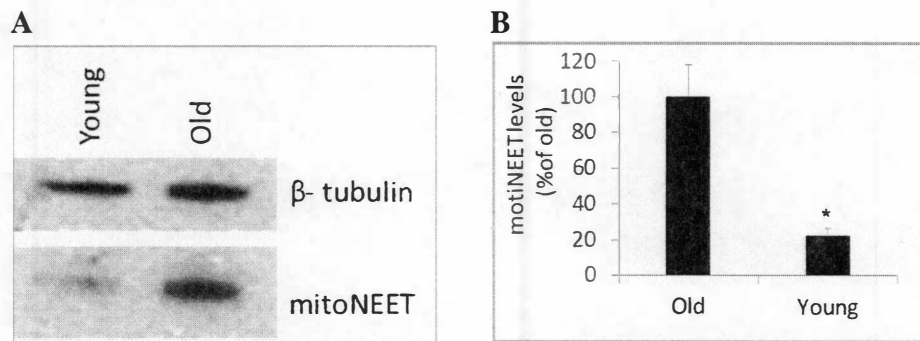


Figure 9. Old mice express higher mitoNEET levels in brain. (A) Western blot showing mitoNEET levels in synaptosomes of young and old mice. (B) Band intensities were quantified using ImageJ software ($p < 0.05$, young $n = 4$, old $n = 3$, $\pm$ SEM).

*statistically significant differences between young and old group of mice.

DISCUSSION

The results of this study document that exposure of HepG2 cells to galactose decreased endogenous mitoNEET levels and the level of the chimeric protein mNT-GFP. Furthermore, treatment of hepatocytes with pioglitazone, which binds to mitoNEET, also reduced endogenous mitoNEET levels at high concentration but restored galactose induced reductions in endogenous mitoNEET levels at low concentration.

Recent studies suggest that, similar to mitoNEET knockdown experiments, chronic administration of galactose facilitates the aging process by increasing oxidative stress, mitochondrial injury and advanced glycation end products (AGE) *in vivo* (Parameshwaran et al., 2010, Semba et al., 2010, Song et al., 1999). Decreases in mitoNEET levels in HepG2 cells cultured in D-galactose (Fig. 5) might be an example of glycation and degradation or a feedback mechanism to control increased mitochondrial activity due to relief of the Crabtree effect (see Chapter 2) (Crabtree 1929). Pioglitazone partially restored mitoNEET levels (Fig. 8), perhaps by inducing PGC1 α expression and counteracting galactose-induced increased respiration by inhibiting complex I and playing a protective role against galactose-induced aging observed in a rodent model (Prakash and Kumar 2013, Kaundal and Sharma 2010, Hunter et al., 2008). Pioglitazone also plays a protective role against D-galactose-induced mitochondrial oxidative damage and apoptosis through activation of nuclear transcription factor PPAR γ receptors (Prakash and Kumar 2013). Increased ROS production, oxidative damage and mitochondrial dysfunction are the characteristic features of aging. We observed synaptosomes isolated from aged group of mice have higher mitoNEET levels (Fig. 9). Overexpression of mitoNEET might be the positive feedback mechanism of

overexpressed PGC1 α to reduce progressively increased ROS levels down thereby protecting cells and organelles.

In conclusion, results suggest that mitoNEET plays a role in biological as well as galactose increased aging, which may be due to decreased mitoNEET levels thereby increasing ROS levels in the cells. Increases in ROS levels as a results of mitoNEET knockdown and galactose treatment should be evaluated in a rodent model using liver tissues or in the HepG2 cell model. Pioglitazone partially restored galactose induced decreases in mitoNEET levels and perhaps suggests that this might ameliorate galactose toxicity in the cells. Biological aging and galactose induced aging likely utilize distinct mechanisms in terms of mitoNEET effects.

LITERATURE CITED

- Altamirano, C., Illanes, A., Becerra, S., Cairó, J. J., & Gòdia, F. (2006). Considerations on the lactate consumption by CHO cells in the presence of galactose. *Journal of Biotechnology*, 125(4), 547-556.
- Brunmair, B., Staniek, K., Gras, F., Scharf, N., Althaym, A., Clara, R., ... & Fürnsinn, C. (2004). Thiazolidinediones, Like Metformin, Inhibit Respiratory Complex IA Common Mechanism Contributing to Their Antidiabetic Actions? *Diabetes*, 53(4), 1052-1059.
- Crabtree, H. G. (1929). Observations on the carbohydrate metabolism of tumours. *Biochemical Journal*, 23(3), 536.
- García-Ruiz, I., Solís-Muñoz, P., Fernández-Moreira, D., Muñoz-Yagüe, T., & Solís-Herruzo, J. A. (2013). Pioglitazone leads to an inactivation and disassembly of complex I of the mitochondrial respiratory chain. *BMC Biology*, 11(1), 88.
- Hendricks, E. W. (2015). The Impact of Aging and Estrogen Therapy on Synaptic Mitochondrial Bioenergetics in an Alzheimer's Mouse Model (Master's Thesis, Eastern Illinois University).
- Hondares, E., Mora, O., Yubero, P., de la Concepción, M. R., Iglesias, R., Giralt, M., & Villarroya, F. (2006). Thiazolidinediones and rexinoids induce peroxisome proliferator-activated receptor-coactivator (PGC)-1 α gene transcription: an autoregulatory loop controls PGC-1 α expression in adipocytes via peroxisome proliferator-activated receptor- γ coactivation. *Endocrinology*, 147(6), 2829-2838.

- Hunter, R. L., Choi, D. Y., Ross, S. A., & Bing, G. (2008). Protective properties afforded by pioglitazone against intrastriatal LPS in Sprague–Dawley rats. *Neuroscience Letters*, 432(3), 198-201.
- Kaundal, R. K., & Sharma, S. S. (2010). Peroxisome proliferator-activated receptor gamma agonists as neuroprotective agents. *Drug News & Perspectives*, 23(4), 241-256.
- Kersten, S., Desvergne, B., & Wahli, W. (2000). Roles of PPARs in health and disease. *Nature*, 405(6785), 421-424.
- Knouff, C., & Auwerx, J. (2004). Peroxisome proliferator-activated receptor- γ calls for activation in moderation: lessons from genetics and pharmacology *Endocrine Reviews*, 25(6), 899-918.
- Kusminski, C. M., Holland, W. L., Sun, K., Park, J., Spurgin, S. B., Lin, Y., ... & Scherer, P. E. (2012). MitoNEET-driven alterations in adipocyte mitochondrial activity reveal a crucial adaptive process that preserves insulin sensitivity in obesity. *Nature Medicine*, 18(10), 1539-1549.
- Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., & Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). *Journal of Biological Chemistry*, 270(22), 12953-12956.
- Lei, M., Hua, X., Xiao, M., Ding, J., Han, Q., & Hu, G. (2008). Impairments of astrocytes are involved in the d-galactose-induced brain aging. *Biochemical and biophysical Research Communications*, 369(4), 1082-1087.

- Parameshwaran, K., Irwin, M. H., Steliou, K., & Pinkert, C. A. (2010). D-galactose effectiveness in modeling aging and therapeutic antioxidant treatment in mice. *Rejuvenation Research*, 13(6), 729-735.
- Prakash, A., & Kumar, A. (2013). Pioglitazone alleviates the mitochondrial apoptotic pathway and mito-oxidative damage in the d-galactose-induced mouse model. *Clinical and Experimental Pharmacology and Physiology*, 40(9), 644-651.
- Rabøl, R., Larsen, S., Højberg, P. M. V., Almdal, T., Boushel, R., Haugaard, S. B., ... & Dela, F. (2010). Regional anatomic differences in skeletal muscle mitochondrial respiration in type 2 diabetes and obesity. *The Journal of Clinical Endocrinology & Metabolism*, 95(2), 857-863.
- Sahin, E., & DePinho, R. A. (2010). Linking functional decline of telomeres, mitochondria and stem cells during ageing. *Nature*, 464(7288), 520-528.
- Salem, A. F., Whitaker-Menezes, D., Howell, A., Sotgia, F., & Lisanti, M. P. (2012). Mitochondrial biogenesis in epithelial cancer cells promotes breast cancer tumor growth and confers autophagy resistance. *Cell Cycle*, 11(22), 4174-4180.
- Schalkwijk, C. G., Stehouwer, C. D., & van Hinsbergh, V. W. (2004). Fructose-mediated non-enzymatic glycation: sweet coupling or bad modification. *Diabetes/Metabolism Research and Reviews*, 20(5), 369-382.
- Semba, R. D., Nicklett, E. J., & Ferrucci, L. (2010). Does accumulation of advanced glycation end products contribute to the aging phenotype? *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 65(9), 963-975.
- Singh, R., Barden, A., Mori, T., & Beilin, L. (2001). Advanced glycation end-products: a review. *Diabetologia*, 44(2), 129-146.

- Song, X. U., Bao, M., Li, D., & Li, Y. M. (1999). Advanced glycation in D-galactose induced mouse aging model. *Mechanisms of Ageing and Development*, 108(3), 239-251.
- Tait, S. W., & Green, D. R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology*, 11(9), 621-632.
- Tamir, S., Paddock, M. L., Darash-Yahana-Baram, M., Holt, S. H., Sohn, Y. S., Agranat, L., ... & Nechushtai, R. (2015). Structure–function analysis of NEET proteins uncovers their role as key regulators of iron and ROS homeostasis in health and disease. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1853(6), 1294-1315.
- Tang, T., & He, B. (2013). Treatment of D-galactose induced mouse aging with Lycium barbarum polysaccharides and its mechanism study. *African Journal of Traditional, Complementary and Alternative Medicines*, 10(4), 12-17.
- Wiley, S. E., Murphy, A. N., Ross, S. A., van der Geer, P., & Dixon, J. E. (2007). MitoNEET is an iron-containing outer mitochondrial membrane protein that regulates oxidative capacity. *Proceedings of the National Academy of Sciences*, 104(13), 5318-5323.
- Willson, T. M., Lambert, M. H., & Kliewer, S. A. (2001). Peroxisome proliferator-activated receptor γ and metabolic disease. *Annual Review of Biochemistry*, 70(1), 341-367.

CHAPTER TWO

THE EFFECTS OF PIOGLITAZONE ON LIVER CELL BIOENERGETICS

ABSTRACT

Insulin resistance is linked to mitochondrial dysfunction and decreased oxidative capacity of metabolically active tissues. Pioglitazone is commonly used to treat type-II diabetes and has been shown to interact with mitoNEET (mNT, CISD1) which localizes to the mitochondrion. We recently observed that human hepatocellular carcinoma cells (HepG2) cultured in presence of D-galactose (10 mM) at low glucose concentrations (<1 mM) express significantly reduced levels of mitoNEET compared to cells cultured in presence of glucose alone. We hypothesized that since galactose and pioglitazone alter cellular mitoNEET levels, significant changes in cellular bioenergetics may occur. We employed respirometry and calorimetry to investigate the effects of mitoNEET levels on cellular bioenergetics after galactose and pioglitazone treatment in HepG2 cells. Respiration rates of permeabilized cells in the presence of mitochondrial substrates (malate, glutamate plus pyruvate) utilized by complex I and ADP (OXPHOS) were significantly increased for HepG2 cells cultured in galactose ($45.8 \pm 1.96 \text{ pmol O}_2 \cdot \text{s}^{-1} \cdot 10^{-6} \text{ cells}$; $n = 6$, $\pm \text{SE}$) compared to cells cultured in 15 mM glucose alone ($30.5 \pm 2.94 \text{ pmol O}_2 \cdot \text{s}^{-1} \cdot 10^{-6} \text{ cells}$; $n = 6$, $\pm \text{SE}$). Acute exposure to 60 μM pioglitazone caused a severe reduction in OXPHOS for cells cultured in either medium (galactose: $12.18 \pm 1.37 \text{ pmol O}_2 \cdot \text{s}^{-1} \cdot 10^{-6} \text{ cells}$; glucose: $10.1 \pm 1.01 \text{ pmol O}_2 \cdot \text{s}^{-1} \cdot 10^{-6} \text{ cells}$).

Overall heat flow of intact cells was $-55.6 \pm 3.0 \text{ }\mu\text{W}$ per million cells in glucose medium and metabolic activity was significantly reduced to $-47.2 \pm 2.5 \text{ }\mu\text{W}$ per million cells ($n = 10$, $\pm \text{SE}$) in cells cultured in the presence of galactose. Surprisingly, acute

treatment of cells with 60 μ M pioglitazone increased heat dissipation in cells grown in glucose medium by ~20%, but the effect was significantly lower for cells cultured in the presence of galactose. Transfection of HepG2 cells with green fluorescent protein tagged mitoNEET (HepG2-GFP-mNT) increased respiration rates and heat flow in either glucose or galactose media. Our results demonstrate that pioglitazone targets complex I of the mitochondrial OXPHOS machinery, but the decrease in mitochondrial efficiency is likely compensated by an increase in glycolytic flux.

INTRODUCTION

Diabetes related complications are the primary cause of death in the USA. In 2012, 29.1 million Americans or 9.3% of the total population have been diagnosed with type II diabetes. Even more strikingly, 11.8 million or 25.9% of elderly people above the age of 65 years have diabetes (National Diabetes Statistics Report, 2014). The American Diabetes Association (ADA) states that diabetes caused 231,404 deaths as listed on the death certificates in 2013 (ADA). Pioglitazone (brand name 'Actos'), PPAR γ agonist, is used to increase the response of cells to the blood-sugar regulating hormone insulin (peripheral tissue insulin sensitivity), and to inhibit the release of blood sugar (glucose) from the liver in type-2 diabetes (Miyazaki 2002). Recent studies demonstrated that pioglitazone also binds to mitoNEET and addresses the underlying cause of type-2 diabetes through increasing deposition of fat (lipids) in fat cells termed adipocytes (Kusminski et al., 2012, Colca et al., 2004).

The biological function(s) of mitoNEET are still unknown, but the proposed functions include donation of the iron-sulfur cluster to unknown acceptor protein(s) and functioning as electron transfer protein. Regulation of redox reactions and oxidative phosphorylation (OXPHOS) in the mitochondria, accumulation of reactive oxygen species (ROS) and control of apoptosis are also the proposed functions of mitoNEET (Tait and Green 2010, Wiley et al., 2007, Kaundal and Sharma 2010). Transfer of unique iron-sulfur cluster of mitoNEET is decisive for mitoNEET functioning (Tamir et al., 2014, Wiley et al., 2007). Binding of pioglitazone to mitoNEET has been shown to reduce the likelihood of iron-sulfur cluster transfer *in vitro* (Paddock et al., 2007, Wiley et al., 2007). High mitoNEET levels are associated with enhanced mitochondrial iron

content, increased respiration rates and reduced weight gain on high fat diet in a mouse model of obesity. Similarly, increased oxidative stress and glucose intolerance are associated with reduced level of mitoNEET (Kusminski et al., 2012).

The major function of mitochondria is to generate and supply ATP to the cells in addition to supplying intermediates for several biosynthetic pathways. Furthermore, mitochondria are the major producers of cellular ROS. Compromised energy metabolism and mitochondrial dysfunction are characteristic features of metabolic diseases like diabetes (Kim et al., 2008), and neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (Swerdlow 2009).

The efficiency of mitochondrial OXPHOS can be defined as the ratio of ATP generated per amount of oxygen consumed (P/O ratio). A high P/O ratio indicates better coupling of the electron transport system (ETS) with the phosphorylation system. Decreases in the proton motive force (Δp) and increased uncoupling of the OXPHOS system leads to waste of redox energy and increases in thermogenesis. Unspecific proton leak (LEAK respiration), fatty acids, uncoupling proteins (UCP), calcium uniport, adenine nucleotide antiport (ADP/ATP exchange), phosphate symport ($\text{PO}_4^{4-}/\text{H}^+$ uptake), and aspartate/glutamate + H^+ exchange are the major mitochondrial membrane potential ($\Delta\Psi_m$) consuming processes across the mitochondrial membrane (Kadenbach 2003)

Experimentally, the state of oxygen consumption by isolated mitochondria after addition of different substrate are traditionally categorized into five states, designated 1 through 5. State 1 is the respiration of mitochondria alone (in presence of P_i) and State 2 includes the addition of ADP without exogenous substrate. State 3 includes the ADP-stimulated oxygen consumption by intact mitochondria in presence of excess substrate (OXPHOS)

respiration and state 4 is carried out in presence of specific mitochondrial substrate (malate, glutamate plus pyruvate, succinate and fatty acids) but without ADP or any metabolic inhibitors (LEAK). Lastly, state 5 is the respiration without oxygen. Only respirations states 3 and 4 are frequently used when measuring the efficiency of mitochondrial OXPHOS. State 3 respiration in presence of substrate plus ADP is often denoted by state 3_{ADP} and in presence of uncoupling reagent is denoted by state 3_u (Nicholls and Ferguson 2013).

The respiration in response to ADP measures the ability of mitochondria to synthesize ATP and is represented by the mitochondrial respiratory control ratio (RCR). The RCR, the proton leak rate (LEAK), the maximum respiratory rate, and the coupling efficiency all provide information about mitochondrial function and dysfunction (Brand and Nicholls 2011). The ratio of state 3/state 4 respiration is known as the RCR. RCR is not an absolute value, but depends on the tissue type and substrate. A high RCR value indicates better membrane integrity, greater capacity for oxidation of substrate and high efficiency in ATP production. State 3 respiration rates depend on the size of cytochrome c-ubiquinone (cyt c-UQ) pool, and concentration of available oxygen, whereas proton leak and ATPase contamination primarily control state 4 respiration (Nicholls and Ferguson 2013). Oligomycin A inhibits the ATP synthase (Salomon et al., 2000). Therefore, a high state 4 respiration rate after addition of oligomycin (state 4o) indicates high proton leak *in vivo* or injury to the inner mitochondrial membrane during the isolation process. A low state 4o indicates high proton motive force (PMF) sufficient enough to restrict electron transport (Brand and Nicholls 2011). PMF (Δp) is defined as a proton circuit across the inner mitochondrial membrane created by proton pumps of ETS

together with ATP synthase. This energetic force or potential is central to mitochondrial bioenergetics which has two components, pH gradient and the membrane potential ($\Delta\Psi_m$) between mitochondrial matrix and cytoplasm (eqn 1) (Brand and Nicholls 2011, Nicholls and Ferguson 2013).

$$\Delta p = \Delta\Psi_m - 61.5 \log_{10}\Delta pH \quad (\text{Equation 1})$$

Warburg effect and Crabtree effect

Glucose is a major source of cellular energy. When the cell has an adequate supply of metabolic energy available in the form of ATP, the breakdown of glucose is inhibited because NADH, generated in the Krebs's cycle, inhibits isocitrate dehydrogenase (EC 1.1.1.41) and α -ketoglutarate dehydrogenase (EC 1.2.4.2), thus accumulating acetyl CoA and lactic acid (Berg et al., 2002). An important aspect of cancer cells is their ability to survive under hypoxic and acidic conditions (Marroquin et al., 2007). Many highly proliferative cells, including cancerous cells, depend on glycolysis for ATP generation despite the abundance of oxygen and fully functional mitochondria (Warburg effect), likely to increase production of precursors for biosynthetic pathways (Heiden et al., 2009). However, despite the reduction in cellular respiration the mitochondria are not impaired, they are just not utilized; this phenomenon was first identified by Otto Warburg and was termed the Warburg Effect (Warburg 1956, Vander et al., 2009).

The phenomenon that some cells, in spite of having fully functional mitochondria and abundant oxygen, shift metabolism towards glycolysis in presence of high glucose concentrations, is known as the Crabtree Effect (Marroquin et al., 2007, Crabtree 1929). When using immortalized cell lines (cancer cells) as a model to evaluate mitochondrial function, it is important to understand the consequences of the Warburg and Crabtree

effects. Many mitochondrial toxicants cannot be detected with this cellular model due to limited use of the mitochondria in immortalized cells (Vander et al., 2009). However, some immortalized cell when grown in galactose medium in the absence of glucose favor mitochondrial respiration (OXPHOS) due to relief from the Crabtree effect (Altamirano et al., 2006). To elucidate the role of mitoNEET in galactose and pioglitazone induced changes in liver cell bioenergetics, we used cellular respirometry and calorimetry.

METHODS

Respirometry of HepG2 cells

Respiration was measured at 37 °C using 1×10^6 cells per mL in each chamber of the Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria). Oxygen consumption of permeabilized cells was measured in 2 mL of MiR05 (110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4 , 3 mM MgCl_2 , 0.5 mM EGTA, 0.1% BSA, 20 mM HEPES-KOH, pH 7.1). In order to supply mitochondrial substrates, cells were permeabilized by the addition of digitonin dissolved in Me_2SO at $10 \text{ mg} \cdot \text{mL}^{-1}$ (final concentration $10 \text{ } \mu\text{g} \cdot 10^{-6} \text{ cells}$). This digitonin concentration was found to be sufficient to permeabilize the plasma membrane of HepG2 cells with low impact on the integrity of the outer mitochondrial membrane. Electron flow through complex I was stimulated by adding 2 mM malate, 10 mM glutamate, and 5 mM pyruvate (MGP). To engage the phosphorylation system, 1 mM ADP was added followed by the addition of 10 mM succinate to supply electrons to the ubiquinone pool via succinate dehydrogenase. Leak respiration in presence of ADP and ATP was measured after addition of oligomycin ($2 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$), and contribution of complex I to leak respiration was recorded after addition of rotenone (0.5 μM). Non-mitochondrial oxygen consumption was recorded after addition of 2.5 μM of antimycin A. Oxygen consumption was measured with an Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria) DATLAB software (OROBOROS Instruments) was used for data analysis and acquisition.

Heat Dissipation

An LKB 2277 thermal activity monitor (Bromma, Sweden) was used to measure the heat dissipation of cells in suspension. Static and dynamic calibrations against water

in both ampoules were performed before the heat signal was measured. For the biological experiments, water in the sample ampoule was replaced with 2.5 ml of cell suspension. One ampule was set as the control (DMSO) while the other ampule had 60 μ M pioglitazone. This total suspension contained between 375,000 and 500,000 cells in culture medium equilibrated with 10% CO₂ and 90% air. All calorimetric measurements were performed at 37 °C. Overall heat flow of intact cells was recorded for 2 hours after the ampoule was lowered into the calorimeter.

Cell proliferation

The cells were maintained in a humidified atmosphere of 6.5% CO₂ and 93.5% air at 37 °C. Cells that were used for proliferation data were also incubated in 15, 30, or 60 μ M pioglitazone. To assess the cell proliferation rates, 250,000 cells per well were plated on a 12-well plate. Different concentrations of pioglitazone were added (0-60 μ M) after 24 h that cells were plated. Cells were counted after 24 and 48 h after pioglitazone treatment. Viable cells were counted with a hemocytometer using trypan blue exclusion by diluting samples in a 1:1 ratio with trypan blue.

Data Analysis

Data were analyzed with a one-way analysis of variance (ANOVA) on ranks followed by comparison of experimental groups with the appropriate control group (Holm-Sidak method) or 2-way ANOVA followed by comparison of experimental groups with the appropriate control groups (Holm-Sidak method). SigmaPlot 12.5 (Systat Software Inc., San Jose, CA) and statistical package R (R version 3.1.2 (2014-10-31)) were used for the analyses.

RESULTS

Galactose increased OXPHOS activity

Oxygen consumption rates in the presence of different mitochondrial substrates were recorded for permeabilized HepG2 and HepG2-mNT-GFP cells which were prior to measurements cultured in galactose supplemented medium or high glucose medium.

In the presence of NADH generating substrates malate, glutamate and pyruvate (MGP) utilized by complex I, HepG2 cells cultured in galactose had significantly lower respiration rates (state 4 respiration) compared to controls (HepG2 in glucose). Pioglitazone treatment did not change oxygen consumption rates in either glucose or galactose media (Fig. 10A). Again, in the presence of NADH generating substrates (MGP), HepG2-mNT-GFP cells cultured in galactose had significantly higher respiration rates compared to their glucose counterparts. Pioglitazone significantly increased state 4 respirations in HepG2-mNT-GFP cells cultured only in glucose medium (Fig. 10A).

The addition of ADP to the conditions described in Fig. 10A resulted in oxygen consumption rates that were significantly increased in both HepG2 and HepG2-mNT-GFP cells compared to their MGP respiration rates. These elevated respiration rates were significantly higher in galactose treated HepG2 cells compared to their glucose counterpart. Elevation of respiration after addition of ADP in HepG2-mNT-GFP cells was not significantly different in either medium. However, HepG2-mNT-GFP cells cultured in both glucose and galactose media showed significantly higher respiration rates compared to HepG2 cells cultured in glucose medium (Fig. 10B).

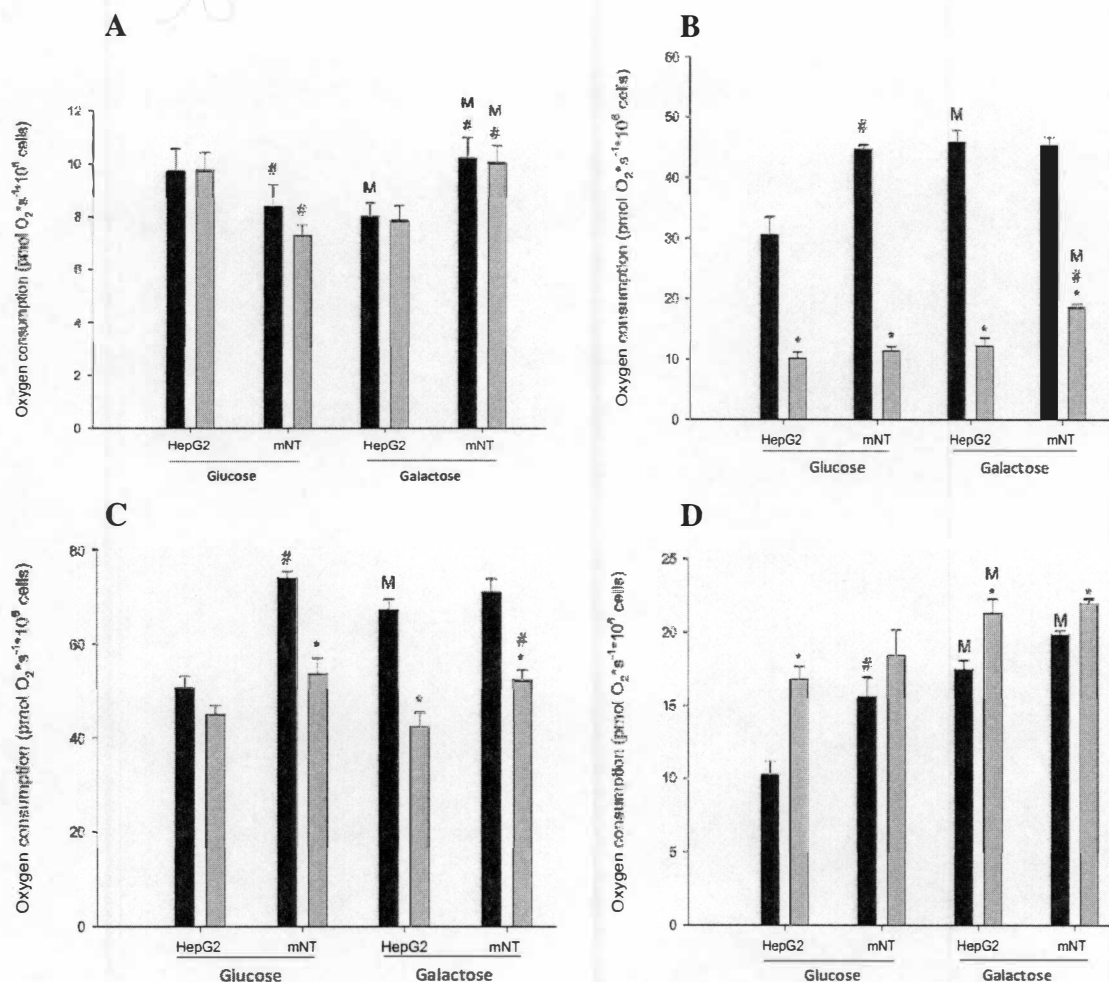


Figure 10. Oxygen consumption of permeabilized HepG2 and HepG2-mNT-GFP (mNT) cells cultured in glucose and DMEM plus galactose (Galactose) in response to acute addition of 60 μ M pioglitazone (gray bar) in the presence of (A) NADH generating substrates (MGP) without ADP, (B) MGP plus ADP, (C) MGP, ADP and succinate and (D) MGP, ADP and succinate (LEAKo respiration) ($n = 6$, $\pm$ SE)

Statistically significant differences in pioglitazone treatment (*), differences between cell lines in the same medium (#) and differences between the media of the same cell line (^M).

Pioglitazone significantly reduced complex I activity

Oxygen consumption rates were recorded in HepG2 and HepG2-mNT-GFP cells after treatment with pioglitazone at the concentration of 60 μ M in presence of NADH generating substrates (MGP) and ADP. Prior to experimentation, those cells were cultured in 10 mM D-galactose supplemented medium. Pioglitazone treatment markedly reduced oxygen consumption rates of permeabilized HepG2 cells and HepG2-mNT-GFP cells cultured in either glucose or in galactose medium. Interestingly, HepG2-mNT-GFP cells cultured in galactose were less sensitive to pioglitazone compared to all other conditions evaluated (Fig. 10B).

Addition of succinate to the conditions shown in Fig.10B generate NADH and FADH₂ utilized by both complexes. The succinate stimulated respiration rates in HepG2 cells cultured in glucose in presence of MGP and ADP increased by an average of 64.5% and that of in galactose medium increased by 45.6%. Similarly, respiration in HepG2-mNT-GFP cells cultured in glucose medium increased by 62.2% and that of galactose increased by 57.5%. Both cell types HepG2 and HepG2-mNT-GFP were treated with pioglitazone at the concentration of 60 μ M. Strikingly, after treatment with pioglitazone, the succinate stimulated respiration rate in presence MGP and ADP increased by 350% in HepG2 cells cultured in glucose medium and that of in galactose increased by 83.3%. Likewise, succinate stimulated respiration increased by 381.8% in HepG2-mNT-GFP cultured in glucose medium and that of in galactose medium increased by 222.2%. Although, succinate stimulated respiration in pioglitazone treated cells increased by an average of 259%, this increase is 19.7% less than the respiration in cells without pioglitazone treatment (Fig. 10C).

D-galactose and pioglitazone increased state 4o (LEAKo) respiration

The addition of oligomycin to the conditions described in Fig. 10C (MGP+ADP+succinate) allowed state 4o (LEAKo) respiration to be evaluated. HepG2 cells and HepG2-mNT-GFP cells cultured in galactose had significantly higher state 4o respiration compared to their control counterparts cultured in glucose medium. Both HepG2 and HepG2-mNT-GFP cells were treated with pioglitazone at the concentration 60 μ M. Pioglitazone treatment significantly increased state 4o respiration across all cultured conditions. Interestingly, pioglitazone treated HepG2 and HepG2-mNT-GFP cells cultured in galactose medium had further increased state 4o respiration compared to their glucose counterparts treated with pioglitazone (Fig. 10D).

MitoNEET decreased glutamate dehydrogenase activity after galactose treatment

Addition of glutamate as the only mitochondrial substrate to permeabilized HepG2 cells before uncoupling allows evaluating the activity of glutamate dehydrogenase. Interestingly, galactose treatment for 2 weeks prior to respirometry lead to a decrease in oxygen consumption in HepG2-mNT-GFP cells compared to respiration in HepG2 cells (Fig. 11A). FCCP (Carbonyl cyanide p-[rifluoromethoxyl]-phenyl-hydrozone) is a pure uncoupler (an ionophore) which dissipates the chemiosmotic gradient leading to loss of regulation of the electron transport system. Maximum uncoupled rates of intact cells were obtained using FCCP. A significant increase in uncoupled respiration was observed in HepG2 cells cultured in galactose medium. However, the uncoupled respiration was significantly reduced in HepG2-mNT-GFP cells cultured in galactose compared to their control counterpart cultured in glucose (Fig. 11B).

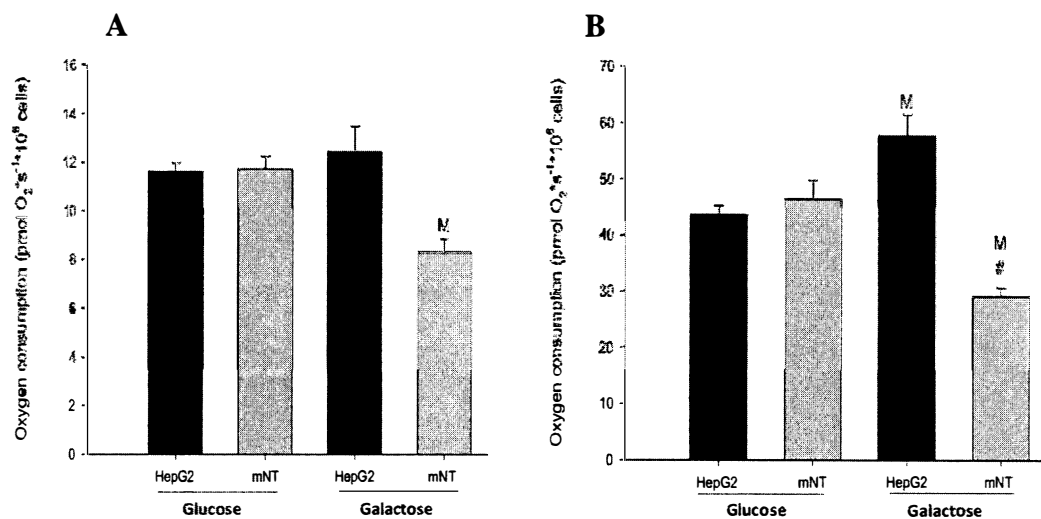


Figure 11. Oxygen consumption of permeabilized HepG2 and HepG2-mNT-GFP (mNT) cells cultured in glucose or galactose (A) using glutamate as the sole substrate, (B) using glutamate as the sole substrate and uncoupling with FCCP ($n = 6$, $\pm$ SE). Statistically significant differences between cell lines in the same medium ([#]) and differences between the media of the same cell line (^M).

Pioglitazone treatment at the concentration of 60 μ M did not show significant uncoupled respiration in HepG2 cells cultured in either medium glucose or galactose. However, pioglitazone treatment significantly increased uncoupled respiration in HepG2-mNT-GFP cells cultured in galactose compared to HepG2 cells cultured in similar conditions (Fig. 12).

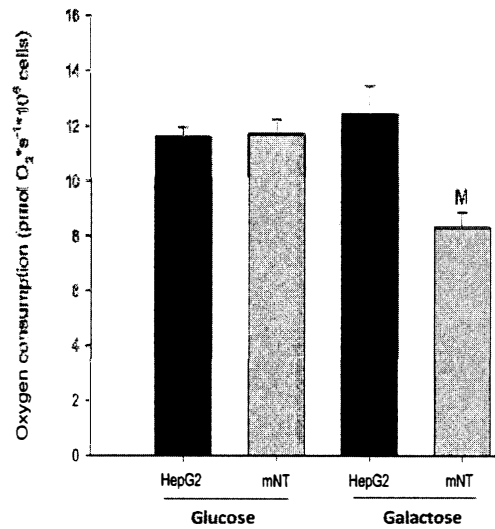


Figure 12. Oxygen consumption of intact HepG2 and HepG2-mNT-GFP (mNT) cells in response to acute addition of 60 μ M pioglitazone during uncoupling of the OXPHOS system. ($n = 6$, $\pm$ SE). Statistically significant differences between the media of the same cell line (^M).

Heat dissipation

Galactose reduced energy transduction

HepG2 cells and HepG2-mNT-GFP cells maintained in high glucose medium were transferred to low glucose containing medium supplemented with 10 mM galactose 2 weeks prior to experimentation and heat flow was evaluated. The results revealed that galactose significantly reduced the heat output in both the HepG2 and HepG2-mNT-GFP cells compared to their control counterparts cultured in glucose medium.

Pioglitazone increased energy transduction over time in galactose supplemented medium

HepG2 and HepG2-mNT-GFP cells cultured in high glucose medium and galactose medium were treated with 60 μ M pioglitazone and heat dissipation was monitored every 30 minutes after treatment for 2 hours. Pioglitazone increased heat dissipation immediately after treatment (30 min) in HepG2 and HepG2-mNT-GFP cells cultured in glucose medium. However, unlike cells cultured in glucose medium, pioglitazone treatment did not increase heat dissipation of cells cultured in galactose 30 min after treatment. However, the heat flow measured 2h after treatment was

Table 1: Cells were cultured and evaluated in either galactose (GAL) or glucose (GLU) based medium. Cells were treated with acute addition of 60 μ M of pioglitazone or DMSO (vehicle control). Overall heat dissipation was increased after addition of pioglitazone to cells in glucose medium but not for galactose based medium ($n=8$, $\pm$ SE).

Condition	30 min	60 min	90 min	120 min
HepG2	55.6 $\pm$ 3.00	56.7 $\pm$ 2.11	55.3 $\pm$ 2.02	53.9 $\pm$ 2.12
HepG2 + Pio	65.5 $\pm$ 2.38	62.9 $\pm$ 2.62	61.5 $\pm$ 2.33	60.0 $\pm$ 2.05
HepG2-Gal	47.1 $\pm$ 2.49	38.7 $\pm$ 1.55	33.6 $\pm$ 1.40	29.9 $\pm$ 1.58
HepG2-Gal + Pio	46.6 $\pm$ 2.47	40.6 $\pm$ 1.80	37.2 $\pm$ 1.50	34.4 $\pm$ 1.29

significantly higher than 30 min (Table 1).

Repeated two-way ANOVA showed significant interactions between time alone (T), media composition alone (M) and the interaction between time, media and pioglitazone treatment on cellular energy transduction (Table 2). The heat generated per million cells measured every 30 minutes and monitored for 2 h had a strong positive correlation to each other (Table 3).

Table 2: Univariate Type III Repeated-Measures ANOVA Assuming Sphericity of change in cellular heat dissipation after galactose and pioglitazone treatment

Source of variation	DF	F	P
Treatment (T)	1,32	0.78	0.38
Media (M)	1,32	43.68	<0.0001***
T $\times$ M	1,32	0.87	0.35
T $\times$ Time	3, 96	3.94	<0.05*
M $\times$ Time	3, 96	33.59	<0.0001***
T $\times$ M $\times$ Time	3, 96	3.29	<0.05*

Table 3: Pairwise correlation analyses change in cellular heat dissipation after galactose and pioglitazone treatment

	30 Min	60 Min	90 Min	120 Min
30 Min	1	0.89	0.85	0.82
60 Min		1	0.99	0.97
90 Min			1	0.99
120 Min				1

DISCUSSION

The results of the present study show that both treatment with D-galactose, (a simple reducing sugar) and mitoNEET overexpression significantly increased OXPHOS capacity in HepG2 cells. Furthermore, treatment of hepatocytes with pioglitazone significantly reduced OXPHOS (state 3) respiration. Interestingly, galactose treatment, pioglitazone treatment and mitoNEET overexpression each increased LEAKo respiration in HepG2 cells.

Mitochondrial respiration is regulated by molecular redox state, the mitochondrial membrane potential ($\Delta\Psi_m$) and proton motive force (PMF/ Δp), state of phosphorylation of ATP/ADP, ion gradient and cellular heat flow in response to intracellular and extracellular energy demands. Mitochondrial respiration in association with cytoplasmic glycolytic pathway provides comprehensive insight of cellular pathophysiology (Pesta and Gnaiger 2012).

We found that D-galactose reduces endogenous mitoNEET levels in HepG2 cells (Fig. 4). Reduction in mitoNEET levels increases iron content in the mitochondrial matrix resulting an enhanced mitochondrial respiration capacity (Kusminski et al., 2012). Immortalized cells grown in glucose continuously produce lactate, which inhibits mitochondrial respiration. However, cells in galactose supplemented medium consume endogenous lactate (Altamirano et al., 2006) favoring mitochondrial respiration. A decreased mitoNEET level and the subsequent increase in mitochondrial iron content combined with endogenous lactate consumption might be the reason for increased respiration in HepG2 cells cultured in galactose medium (Fig. 10).

Overexpression of mitoNEET reduces $\Delta\Psi_m$ in adipocytes and decreases iron content in the mitochondrial matrix thereby increasing reactive oxygen species. Decreased $\Delta\Psi_m$ is associated with insufficient electron transportation system (ETS) activity, inefficient substrate supply to the mitochondria or an increase in proton flux back into the mitochondrial matrix. The observed high rate of respiration in mNT cells compared to HepG2 cells (Fig. 10B) might be due to iron induced ROS production (Kusminski et al., 2012, Simcox and McClain 2013).

In addition to ATP generation, free radicals are also generated as a byproduct of OXPHOS in the inner mitochondrial membrane, which is the major source of reactive oxygen species (ROS) production and subsequent cellular damage. This process is highly dependent on $\Delta\Psi_m$. Electrons are leaked from complex I and complex III to the oxygen producing superoxide radicals. This facilitated proton diffusion is termed “proton leak”. Complex I generates superoxide in the mitochondrial matrix which is believed to contribute much more than complex III to ROS production, whereas complex III generates ROS in both the inter membrane space and the matrix (Jastroch et al., 2010). Additionally, α -ketoglutarate dehydrogenase also contributes to superoxide generation in the mitochondrial matrix (Starkov et al., 2004). Thermogenesis is also a major functions of LEAK respiration in adipocytes which is mediated by uncoupling proteins (UCPs) increasing proton leak and maintaining carbon flux despite low ATP demand in the cells. According to the uncoupling to survive hypothesis, proton leak has been considered as a pathway to minimize oxidative damage by moderating PMF and ROS production. Increased leak respiration in galactose and pioglitazone treated cells in this experiment (Fig. 10D) might be a mechanism to minimize oxidative damage adjusting $\Delta\Psi_m$ and

mitochondrial superoxide production (uncoupling to survive) (Divakaruni and Brand 2011, Jastroch et al., 2010).

Pioglitazone reduces the activity of the mitochondrial complex I by disassembling complex I subunits thereby depleting cellular ATP content. Genes encoding mitochondrial complex I subunits and complex III subunits are upregulated as a result of cellular ATP depletion in HepG2 cells and in mouse liver mitochondria (García-Ruiz et al., 2013). Pioglitazone inhibited state 3 respiration observed in this experiment (Fig. 10B) is perhaps due to disassembly of complex I subunits.

Galactose treatment induces oxidative stress and mitochondrial damage thereby dissipating the electrochemical gradient across the inner mitochondrial membrane, causing a reduction in ATP synthesis (López-Erauskin et al., 2012) leading to a reduction in cell proliferation rates and lower carbon flow. This reduced carbon flow might be the cause behind low heat dissipation in HepG2 cells cultured in galactose medium (Table 1). In conclusion, three significant findings from these experiments include 1) OXPHOS increases due to galactose treatment or mitoNEET overexpression in liver cancer cells, 2) Pioglitazone significantly reduces complex I respiration irrespective of mitoNEET levels and 3) and galactose reduces overall energy transduction in liver cancer cells.

LITERATURE CITED

- Altamirano, C., Illanes, A., Becerra, S., Cairó, J. J., & Gòdia, F. (2006). Considerations on the lactate consumption by CHO cells in the presence of galactose. *Journal of Biotechnology*, 125(4), 547-556.
- Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002). Glycolysis is an energy-conversion pathway in many organisms. *Biochemistry*, 5th edition. New York: W H Freeman; Section 16.1, retrieved on 11/01/2014, Available from: <http://www.ncbi.nlm.nih.gov/books/NBK22593/>
- Brand, M. D. (2005). The efficiency and plasticity of mitochondrial energy transduction. *Biochemical Society Transactions*, 33(5), 897-904.
- Brand, M., & Nicholls, D. (2011). Assessing mitochondrial dysfunction in cells. *Biochem. J*, 435, 297-312.
- Centers for Disease Control and Prevention. (2014). National diabetes statistics report: estimates of diabetes and its burden in the United States, 2014. *Atlanta, GA: US Department of Health and Human Services*. Retrieved on 5-31-2015 from: <http://www.cdc.gov/diabetes/pubs/statsreport14/national-diabetes-report-web.pdf>
- Colca, J. R., McDonald, W. G., Waldon, D. J., Leone, J. W., Lull, J. M., Bannow, C. A., ... & Mathews, W. R. (2004). Identification of a novel mitochondrial protein ("mitoNEET") cross-linked specifically by a thiazolidinedione photoprobe. *American Journal of Physiology-Endocrinology and Metabolism*, 286(2), E252-E260.
- Divakaruni, A. S., & Brand, M. D. (2011). The regulation and physiology of mitochondrial proton leak. *Physiology*, 26(3), 192-205.

- García-Ruiz, I., Solís-Muñoz, P., Fernández-Moreira, D., Muñoz-Yagüe, T., & Solís-Herruzo, J. A. (2013). Pioglitazone leads to an inactivation and disassembly of complex I of the mitochondrial respiratory chain. *BMC Biology*, *11*(1), 88.
- Jastroch, M., Divakaruni, A. S., Mookerjee, S., Treberg, J. R., & Brand, M. D. (2010). Mitochondrial proton and electron leaks. *Essays Biochem*, *47*, 53-67.
- Kadenbach, B. (2003). Intrinsic and extrinsic uncoupling of oxidative phosphorylation. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1604*(2), 77-94.
- Kaundal, R. K., & Sharma, S. S. (2010). Peroxisome proliferator-activated receptor gamma agonists as neuroprotective agents. *Drug News & Perspectives*, *23*(4), 241-256.
- Kim, J. A., Wei, Y., & Sowers, J. R. (2008). Role of mitochondrial dysfunction in insulin resistance. *Circulation Research*, *102*(4), 401-414.
- Kusminski, C. M., Holland, W. L., Sun, K., Park, J., Spurgin, S. B., Lin, Y., ... & Scherer, P. E. (2012). MitoNEET-driven alterations in adipocyte mitochondrial activity reveal a crucial adaptive process that preserves insulin sensitivity in obesity. *Nature Medicine*, *18*(10), 1539-1549.
- López-Erauskin, J., Galino, J., Bianchi, P., Fourcade, S., Andreu, A. L., Ferrer, I., ... & Pujol, A. (2012). Oxidative stress modulates mitochondrial failure and cyclophilin D function in X-linked adrenoleukodystrophy. *Brain*, *135*(12), 3584-3598.
- Marroquin, L. D., Hynes, J., Dykens, J. A., Jamieson, J. D., & Will, Y. (2007). Circumventing the Crabtree effect: replacing media glucose with galactose

- increases susceptibility of HepG2 cells to mitochondrial toxicants. *Toxicological sciences*, 97(2), 539-547.
- Miyazaki, Y., Mahankali, A., Matsuda, M., Mahankali, S., Hardies, J., Cusi, K., ... & DeFronzo, R. A. (2002). Effect of pioglitazone on abdominal fat distribution and insulin sensitivity in type 2 diabetic patients. *The Journal of Clinical Endocrinology & Metabolism*, 87(6), 2784-2791.
- Nicholls, D. G., & Ferguson, S. (2013). *Bioenergetics*. Academic Press. 4th edn, pp. 61–82.
- Paddock, M. L., Wiley, S. E., Axelrod, H. L., Cohen, A. E., Roy, M., Abresch, E. C., ... & Jennings, P. A. (2007). MitoNEET is a uniquely folded 2Fe–2S outer mitochondrial membrane protein stabilized by pioglitazone. *Proceedings of the National Academy of Sciences*, 104(36), 14342-14347.
- Pesta, D., & Gnaiger, E. (2012). High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. In *Mitochondrial Bioenergetics* (pp. 25-58). Humana Press.
- Salomon, A. R., Voehringer, D. W., Herzenberg, L. A., & Khosla, C. (2000). Understanding and exploiting the mechanistic basis for selectivity of polyketide inhibitors of F₀F₁-ATPase. *Proceedings of the National Academy of Sciences*, 97(26), 14766-14771.
- Simcox, J. A., & McClain, D. A. (2013). Iron and diabetes risk. *Cell Metabolism*, 17(3), 329-341.
- Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S., & Beal, M. F. (2004). Mitochondrial α -ketoglutarate dehydrogenase complex

- generates reactive oxygen species. *The Journal of Neuroscience*, 24(36), 7779-7788.
- Swerdlow, R. H. (2009). The neurodegenerative mitochondriopathies. *Journal of Alzheimer's Disease*, 17(4), 737-751.
- Tait, S. W., & Green, D. R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology*, 11(9), 621-632.
- Tamir, S., Paddock, M. L., Darash-Yahana-Baram, M., Holt, S. H., Sohn, Y. S., Agranat, L., ... & Nechushtai, R. (2014). Structure–function analysis of NEET proteins uncovers their role as key regulators of iron and ROS homeostasis in health and disease. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*.
- Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*, 324(5930), 1029-1033.
- Warburg, O. (1956). On the origin of cancer cells. *Science*, 123(3191), 309-314.
- Wiley, S. E., Murphy, A. N., Ross, S. A., van der Geer, P., & Dixon, J. E. (2007). MitoNEET is an iron-containing outer mitochondrial membrane protein that regulates oxidative capacity. *Proceedings of the National Academy of Sciences*, 104(13), 5318-5323.

CHAPTER THREE

CO-IMMUNOPRECIPITATION ASSAY IDENTIFIES BINDING PARTNERS OF MITONEET

ABSTRACT

The recently discovered protein mitoNEET has been shown to play a central role in various metabolic processes including mitochondrial function, programmed cell death (apoptosis), and cell proliferation. However, a clear link between the mechanisms of mitoNEET's functions and the physiological processes with which the protein is involved is still unresolved. A key question that remains is what protein(s) with known function acts as binding partner of mitoNEET. Knowing this would provide a better understanding into the mechanism by which mitoNEET integrates into physiological processes. To detect potential *in vivo* binding partners of mitoNEET, an *in vitro* assay termed co-immunoprecipitation (Co-IP) was performed. A combined approach of the Co-IP assay, proteomics analysis and database search demonstrates that mitoNEET interacts with mitochondrial, cytoplasmic and nuclear proteins including the mitochondrial matrix heat shock proteins 60 (Hsp60), 10 (Hsp10) and aldolase enzymes. These results open new avenues for further research on elucidation of mitoNEET functions and its importance.

INTRODUCTION

Mitochondrial functions and dysfunctions set the capacity of the organelle to supply ATP as per cellular ATP demand (Geldenhuys et al., 2014). The exact cellular functions of the mitoNEET are still unclear but recent reports have been highlighting a role of mitoNEET in various metabolic and neurodegenerative diseases such as type II diabetes, Alzheimer's disease and Parkinson's disease. Furthermore, mitoNEET plays a vital role in mitochondrial bioenergetics, mitophagy, autophagy, apoptosis as well as cellular growth and development (Geldenhuys et al., 2011, Geldenhuys et al., 2014). It has been shown that mitoNEET interacts with other binding partners including ferredoxin and glutamate dehydrogenase 1 maintaining mitochondrial bioenergetics, mitochondrial ROS and iron homeostasis, peripheral tissue insulin sensitivity, apoptosis regulation and mitophagy regulation (Colca et al., 2004, Paddock et al., 2007, Kusminski et al., 2012, , Roberts et al., 2013 and Salem et al., 2012). When mitoNEET is in the oxidized state such as when cells are stressed, mitoNEET transfers its iron-sulfur clusters [2Fe-2S] to an apo-acceptor protein (e.g. ferredoxin), and functions as a redox sensor center (Zuris et al., 2011). Although, the effects of overexpression and knockdown of mitoNEET in insulin sensitivity and ROS homeostasis have been reported in a rodent model (Kusminski et al., 2012) (also see Chapter 2), a clear link between the biochemical characteristics of mitoNEET, and the physiological consequences of mitoNEET levels on mitochondrial performance and oncogenesis is still unresolved. A key question that remains is, what protein(s) with known function(s) works as binding partner of mitoNEET to gain insight into the mechanisms of action of mitoNEET. Our laboratories recently demonstrated the interaction between glutamate dehydrogenase 1 (GDH1), described as a regulator of

insulin, and mitoNEET by forming a redox-sensitive disulfide bond (Roberts et al., 2013). Nevertheless, whether mitoNEET interacts with GDH1 *in vivo* by the same mechanism is unknown. To answer this question, co-immunoprecipitation (CO-IP) assays were performed.

METHODS

Co-immunoprecipitation assay

Thermo Scientific Pierce™ Crosslink IP/Co-IP Kit (88805) was used to perform Co-IP assay following the manufacturer's protocol. Briefly, 2-10µg of anti-CISD1 antibody (Mouse monoclonal [2B3] to CISD1) was cross-linked and immobilized to protein beads provided. Lysis buffer was added to a monolayer of about 5×10^6 HepG2 (ATCC HB-8065, Manassas, VA) cells. After scraping the cells and centrifuging the lysate, the supernatant was applied to the antibody immobilized beads. Elutes were used for SDS-PAGE and Western blot analysis. A pre-cast SDS-PAGE gel (4-15% TGX, Bio Rad, Hercules, CA) was run and the gel was subjected to silver staining using Thermo Scientific Pierce Silver Stain for Mass Spectrometry (#24600) kit following the manufacturer's protocol. Four protein bands were chosen based on their intensity and exhibited apparent molecular weights of approximately 12, 24, 60 and 70 kDa. The four bands were excised from the gel, de-stained using Thermo Scientific Pierce Silver Stain for Mass Spectrometry (24600) kit following the manufacturer's protocol and sent for proteomic analysis to be sequenced and identified.

Proteomic Analysis

All of the proteomic analyses were completed at Indiana University School of Medicine Proteomics Core Laboratory (Indianapolis, IN). The protein samples were digested by trypsin (Promega) overnight at 37°C after de-staining (with 10 mM DTT in 10 mM ammonium bicarbonate) and alkylation (with 55 mM iodoacetamide prepared in 10 mM ammonium bicarbonate) of gel bands. Digested peptides were extracted from the gel spots (with 1) 50% ACN /49.9% H₂O/0.1% TFA; 2) 99.9% ACN/0.1% TFA) and

injected onto NanoAcquity UPLC® column 100µm x 100mm, 1.7µm BEH130 C18 (Analytical column) and NanoAcquity UPLC® Trap column 180µm x 20mm, 5µm Symmetry C18 (Trapping column). A linear gradient of 3 to 40% acetonitrile (in water with 0.1% FA) was used to elute the peptides and effluent was electro-sprayed into the mass spectrometer (Thermo-Fisher Scientific Orbitrap Velos Pro and Waters Acquity UPLC system). Blanks were run prior to the sample run to account for any background signals from solvents or the column. Database search was carried out using Sequest™ algorithms against the latest Human DB from UniProt.

RESULTS

Proteomics analysis and database searches performed after Co-IP assay using anti-CISD1 antibody for the pull-down experiment (Fig. 13) detected 8 different proteins. The majority of proteins identified are localized to the cytoplasm and mitochondrion.

Uniprot database searches for the detected proteins revealed the protein Hsp60 (60 kDa chaperonin) which is localized to the mitochondrial matrix and delta (3,5)-delta(2,4)-dienoyl-CoA isomerase is localized to mitochondria, peroxisomes, extracellular exosomes and membranes. Heterogeneous nuclear ribonucleoprotein A1 is localized to nucleus whereas the THO complex subunit 4 is localized to nucleus, nucleus speckle and cytoplasm and both were identified as potential binding partners of mitoNEET. THO complex, a nuclear

complex containing 5 subunits which is required for transcription elongation through genes comprising tandem repeat DNA sequences.

Furthermore, glyceraldehyde-3-phosphate dehydrogenase (EC:1.2.1.12), fructose-bisphosphate aldolase B (EC:4.1.2.13), and Hsp10 were identified and all proteins localize to the cytoplasm (Table 1).

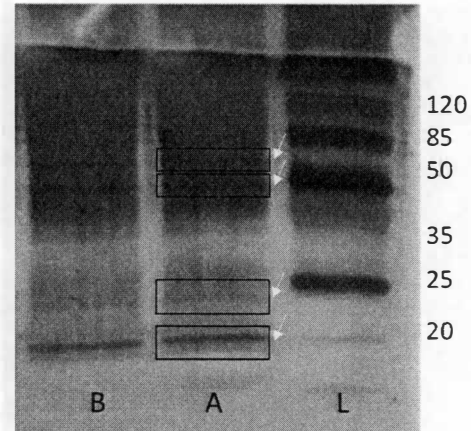


Figure 13. SDS-PAGE gel after silver staining. Proteins isolated from two replicates of HepG2 cells (A and B) were loaded on to the gel. Boxes shown by white arrows were incised and sent to proteomics analysis. Prestained protein serves as protein ladder (L).

Table 4: List of proteins detected using Co-IP, proteomics analysis and database search.

SN	Uniprot ID	Proteins detected	Gene names	Subcellular localization
1	THOC4_HUMAN	THO complex subunit (26.9kDa)	4 <i>ALYREF</i> (<i>ALY, BEF, THOC4</i>)	Nucleus, nucleus speckle, cytoplasm
2	ECH1_HUMAN	Delta (3,5)-delta(2,4)-dienoyl-CoA isomerase, mitochondrial (36 kDa) (EC:5.3.3.)	<i>ECH1</i>	Mitochondria and peroxysome
3	E7EUT5_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase (27.8 kDa) (EC:1.2.1.12)	<i>GAPDH</i>	Cytoplasm and membrane-bound organelle
4	ALDOB_HUMAN	Fructose-bisphosphate aldolase B (39.5 kDa) (EC:4.1.2.13) Liver-type aldolase	<i>ALDOB</i>	Cytoplasm and cytoskeleton
5	H3BPS8_HUMAN	Fructose-bisphosphate aldolase (30.4 (kDa) (EC:4.1.2.13)	<i>ALDOA</i>	Cytoplasm
6	B8ZZL8_HUMAN	10 kDa heat shock protein, mitochondrial/Heat shock 10kDa protein 1 (Chaperonin 10), isoform CRA_b	<i>HSPE1</i>	Cytoplasm/ mitochondria
7	B7Z712_HUMAN	60 kDa heat shock protein (60 kDa chaperonin) (61 kDa)	<i>HSPD1</i>	Mitochondrial matrix
8	H0YH80_HUMAN	Heterogeneous nuclear ribonucleoprotein A1 (19.4 kDa)	<i>HNRNPA1</i>	Nucleus

DISCUSSION

In this study we identified THO complex subunit 4, aldolases, HSP proteins and heterogeneous nuclear ribonucleoprotein A1 as potential binding partner of mitoNEET and the proteins are found in the nucleus, cytoplasm, mitochondria and peroxisomes.

Molecular chaperones bind other nonnative proteins to assist them to fold and gain functional conformation after translation and guide these nonnative proteins to their target organelles. They also participate in refolding of misfolded proteins, assembling and disassembling oligomeric protein structures and regulating biological activities of regulatory proteins including transcription factors. Furthermore, molecular chaperons involve in assisting degradation of unwanted proteins in expense of ATP (Bukau and Horwich 1998). Although diverse classes of molecular chaperones have been reported, heat shock protein 70 (Hsp70) and Hsp60 (chaperonin) are the best characterized ones. But, Hsp60 are quickly sorted into the mitochondria (Itoh et al., 2002). Similar to other Hsps, Hsp70 for example, a complex of mitochondrial Hsp60 and Hsp10 participate in mitochondrial protein folding and their congregation (Nielsen et al., 1999, Campanella et al., 2008). Our results documents potential interaction of mitoNEET with mitochondrial matrix proteins including GDH1 (Roberts et al., 2013) which indicates possible presence of mitoNEET in mitochondrial matrix as well.

The transcribed RNA molecules are transported to the cytoplasm through the nuclear pore complexes via export receptors. Unlike small RNAs (transported via simple export routs), large RNAs (mRNA and rRNA) need complicated ribonucleoproteins (RNP) and adaptor proteins complex for transcription and export (TREX complex) (Köhler and Hurt 2007, Chávez et al 2000). THO complex subunit 4 (THOC4) in

association with other proteins such as ALYREF, mediates the recruitment of TREX complex and nuclear transportation of spliced mRNA. For example, in combination with THOC5, THOC4 aids in the export of spliced HSP70 mRNA from the nucleus (Sträßer et al., 2002). Interaction of mitoNEET with Hsp60, Hsp10, heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and THO subunit 4 likely suggest its transcription, export and folding is mediated by the THO complex/ hnRNP A1 and aided by Hsp60/Hsp10 complexes. Furthermore, mutation in *HNRNPA1* gene which encodes hnRNP A1 causes symptoms similar to neurodegenerative diseases (Benatar et al., 2013) and several recent studies have been reported a crucial role of mitoNEET in neurodegenerative diseases (Sain et al., 2011, Geldenhuys et al., 2011). In addition to molecular chaperones, these Hsp help the cells survive to stressful conditions (Zügel and Kaufmann 1999). Hsp60/Hsp10 complex functions as oxidative sensor center in mitochondria (Samali et al., 1999). Hsp10 has also been reported to play a role in autoimmune inhibition in cancer (Jia et al., 2011).

Several metabolic enzymes were identified in the pull-down assay such as fructose-bisphosphate aldolase (also termed aldolase) an enzyme that reversibly catalyzes degradation of carbohydrates in glycolysis. Similarly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified which catalyzes the sixth step of glycolysis (glyceraldehyde-3phosphate to glyceraldehyde 1,3-biphosphate). Furthermore, GAPDH have been reported to play a role in apoptosis (Tarze et al., 2007). One of the causes of oxidative stress and glucose intolerance phenotypes observed with reduced mitoNEET levels in a rodent model (Kusminski et al., 2012) could be the interactions of mitoNEET with GAPDH and aldolases. Furthermore, mitoNEET lowers β -oxidation rate in a rodent

model (Kusminski et al., 2012) that possibly could be due to an interaction with delta (3,5)-delta(2,4)-dienoyl-CoA isomerase enzyme which catalyzes isomerization of 3-trans,5-cis-dienoyl-CoA to 2-trans,4-trans-dienoyl-CoA in lipid metabolism (fatty acid β -oxidation) pathway (Lou et al., 1994). In conclusion, the Co-IP assay identified 8 potential binding partners of mitoNEET. These proteins have different localizations and functions. To confirm the biological relevance of any of these interactions further research is needed.

LITERATURE CITED

- Benatar, M., Wu, J., Fernandez, C., Weihl, C. C., Katzen, H., Steele, J., ... & Taylor, J. P. (2013). Motor neuron involvement in multisystem proteinopathy Implications for ALS. *Neurology*, 80(20), 1874-1880.
- Bukau, B., & Horwich, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell*, 92(3), 351-366.
- Campanella, C., Bucchieri, F., Ardizzone, N. M., Gammazza, A. M., Montalbano, A., Ribbene, A. N. N. A., ... & Cappello, F. (2009). Upon oxidative stress, the antiapoptotic Hsp60/procaspase-3 complex persists in mucoepidermoid carcinoma cells. *European Journal of Histochemistry*, 52(4), 221-228.
- Chávez, S., Beilharz, T., Rondón, A. G., Erdjument-Bromage, H., Tempst, P., Svejstrup, J. Q., ... & Aguilera, A. (2000). A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in *Saccharomyces cerevisiae*. *The EMBO Journal*, 19(21), 5824-5834.
- Colca, J. R.; McDonald, W. G.; Waldon, D. J.; Leone, J. W.; Lull, J. M.; Bannow, C. A.; Lund, E. T.; Mathews, W. R. Identification of a novel mitochondrial protein ("mitoNEET") cross-linked specifically by a thiazolidinedione photoprobe. *Am. J. Physiol. Endocrinol. Metab.* 2004, 286, 252-260.
- Geldenhuys, W. J., Leeper, T. C., & Carroll, R. T. (2014). mitoNEET as a novel drug target for mitochondrial dysfunction. *Drug discovery today*, 19(10), 1601-1606.

- Geldenhuys, W. J., Youdim, M. B., Carroll, R. T., & Van der Schyf, C. J. (2011). The emergence of designed multiple ligands for neurodegenerative disorders. *Progress in Neurobiology*, 94(4), 347-359.
- Itoh, H., Komatsuda, A., Ohtani, H., Wakui, H., Imai, H., Sawada, K. I., ... & Hamada, F. (2002). Mammalian HSP60 is quickly sorted into the mitochondria under conditions of dehydration. *European Journal of Biochemistry*, 269(23), 5931-5938.
- Jia, H., Halilou, A. I., Hu, L., Cai, W., Liu, J., & Huang, B. (2011). Heat shock protein 10 (Hsp10) in immune-related diseases: one coin, two sides. *International Journal of Biochemistry and Molecular Biology*, 2(1), 47.
- Köhler, A., & Hurt, E. (2007). Exporting RNA from the nucleus to the cytoplasm. *Nature Reviews Molecular Cell Biology*, 8(10), 761-773.
- Kusminski, C. M., Holland, W. L., Sun, K., Park, J., Spurgin, S. B., Lin, Y., Askew, G. R., Simcox, J. A., McClain, D. A., Li, C., & Scherer, P. E. (2012). MitoNEET-driven alterations in adipocyte mitochondrial activity reveal a crucial adaptive process that preserves insulin sensitivity in obesity. *Nature Medicine*, 18(10), 1539-1549.
- Luo, M. J., Smeland, T. E., Shoukry, K., & Schulz, H. (1994). Delta 3, 5, delta 2, 4-dienoyl-CoA isomerase from rat liver mitochondria. Purification and characterization of a new enzyme involved in the beta-oxidation of unsaturated fatty acids. *Journal of Biological Chemistry*, 269(4), 2384-2388.

- Nielsen, K. L., McLennan, N., Masters, M., & Cowan, N. J. (1999). A single-ring mitochondrial chaperonin (Hsp60-Hsp10) can substitute for GroEL-GroES in vivo. *Journal of Bacteriology*, 181(18), 5871-5875.
- Paddock, M. L., Wiley, S. E., Axelrod, H. L., Cohen, A. E., Roy, M., Abresch, E. C., Capraro, D., Murphy, A.N., Nechushtai, R., Dixon, J.E. & Jennings, P. A. (2007). MitoNEET is a uniquely folded 2Fe-2S outer mitochondrial membrane protein stabilized by pioglitazone. *Proceedings of the National Academy of Sciences*, 104(36), 14342-14347.
- Roberts, M. E., Crail, J. P., Laffoon, M. M., Fernandez, W. G., Menze, M. A., & Konkle, M. E. (2013). Identification of disulfide bond formation between mitoNEET and glutamate dehydrogenase 1. *Biochemistry*, 52(50), 8969-8971.
- Sain, H., Sharma, B., Jaggi, A. S., & Singh, N. (2011). Pharmacological investigations on potential of peroxisome proliferator-activated receptor-gamma agonists in hyperhomocysteinemia-induced vascular dementia in rats. *Neuroscience*, 192, 322-333.
- Salem, A. F., Whitaker-Menezes, D., Howell, A., Sotgia, F., & Lisanti, M. P. (2012). Mitochondrial biogenesis in epithelial cancer cells promotes breast cancer tumor growth and confers autophagy resistance. *Cell Cycle*, 11(22), 4174-4180.
- Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., & Orrenius, S. (1999). Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells. *The EMBO Journal*, 18(8), 2040-2048.

- Sträßer, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., ... & Hurt, E. (2002). TREX is a conserved complex coupling transcription with messenger RNA export. *Nature*, 417(6886), 304-308.
- Tarze, A., Deniaud, A., Le Bras, M., Maillier, E., Molle, D., Larochette, N., ... & Brenner, C. (2007). GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization. *Oncogene*, 26(18), 2606-2620.
- Uniport <http://www.uniprot.org/uniprot/> (accessed June 12, 2015)
- Zügel, U., & Kaufmann, S. H. (1999). Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clinical Microbiology Reviews*, 12(1), 19-39.
- Zuris, J. A., Harir, Y., Conlan, A. R., Shvartsman, M., Michaeli, D., Tamir, S., ... & Nechushtai, R. (2011). Facile transfer of [2Fe-2S] clusters from the diabetes drug target mitoNEET to an apo-acceptor protein. *Proceedings of the National Academy of Sciences*, 108(32), 13047-13052.