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Pioglitazone, NEET Family Proteins, and Galactose Modulation of Liver Cell Bioenergetics

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Pioglitazone, NEET Family Proteins, and Galactose Modulation of Liver Cell Bioenergetics

BY

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UNDERGRADUATE THESIS

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Abstract

MitoNEET was discovered through interactions with a labeled and photoactive derivative of pioglitazone (pio), a drug used to increase peripheral insulin sensitivity. Its unique coordination of a [2Fe-2S] cluster by three cysteine residues (Cys-72, Cys-74, and Cys-83) and one histidine (His-87) gives this cluster both stability and the ability to be donated to acceptor proteins. These qualities allow mitoNEET to participate in a diversity of biological functions. Functions of mitoNEET and the consequences of pioglitazone (pio) treatment in human hepatocellular carcinoma (HepG2) cells cultured in glucose or galactose-based medium were examined by respiration and proliferation studies. Pio treatment decreased complex I stimulated respiration for cells grown in both glucose and galactose-based medium. Additionally, pio was found to significantly decrease cell proliferation. HepG2 cells cultured in galactose exhibited significantly higher oxygen flux than those cultured in glucose-based medium, but proliferation of these cells was notably reduced. Interestingly, mitoNEET levels were substantially lower in cells cultured in galactose. We hypothesize that some of the effects of pio may depend on the cellular levels of mitoNEET and the metabolic consequences of culturing cancerous cells in a galactose-based medium.

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Introduction

Type-II diabetes is characterized by diminished insulin secretion and reduced insulin sensitivity¹. Thiazolidinediones (TZDs) (Fig. 1) are a class of drugs that work as glucose-lowering agents and have been reported to impact mitochondrial activity^{1,2}. Pioglitazone, a

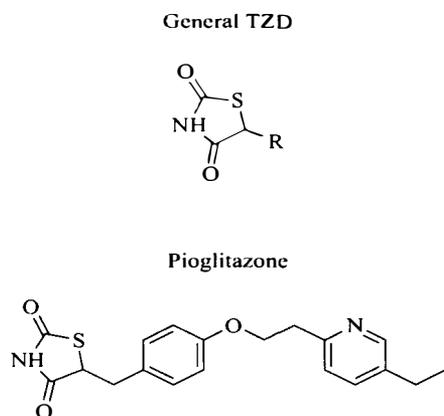


Figure 1. The general structure of thiazolidinediones (TZDs) followed by the structure of pioglitazone. "R" represents any substituent specific to TZDs.

member of this drug class, is used in the treatment of type-II diabetes and was thought to exhibit its therapeutic functions by binding to peroxisome proliferator-activated receptor gamma (PPAR- γ)². This receptor is responsible for several components of metabolic control, namely lipid and glucose metabolism^{3,4}. The therapeutic effects of TZDs are not

limited to type-II diabetes, but may also provide benefit for patients with metabolic syndrome and cardiovascular disease^{5,6}. These effects and others may be mediated through other processes related to the mitochondrion, as pioglitazone was found to inhibit complex I and III of the respiratory chain, induce mitochondrial biogenesis in adipose tissue, and bind with the protein mitoNEET⁷⁻¹⁰.

NEET family proteins are a class of iron-sulfur cluster proteins that contain a unique three cysteine (Cys-72, Cys-74, and Cys-83) and one histidine (His-87) CDGSH domain coordination of the [2Fe-2S] cluster (Fig. 2)¹¹. This coordination provides more stability compared to other classes of [2Fe-2S] cluster proteins, but it is capable of transfer to acceptor proteins^{12,13}. The metal cluster is labile under acidic conditions, which is in stark contrast to other protein families that contain [2Fe-2S] cluster¹⁴. MitoNEET is a small NEET family protein that was discovered through interactions with a labeled and photoactive derivative of the TZD

pioglitazone⁷. Localized to the outer membrane of the mitochondrion mitoNEET has been found to play a significant role in regulating electron transfer and oxidative phosphorylation in mammalian cells¹. Pioglitazone has been shown to stabilize mitoNEET's cluster *in vitro*, but the physiological consequences of these data are undetermined^{7,12}. Other proposed functions of mitoNEET include the regulation of redox reactions and reactive oxygen species in the mitochondria, control of carbon flux through interactions with dehydrogenase enzymes, and the regulation of cellular iron homeostasis^{1,15,16}. In a study examining the role of microRNAs (miRNA) in spinal chord injuries in rats, the targeting of mitoNEET with miRNA resulted in neuronal loss and apoptosis in primary cultured spinal neurons¹⁷.

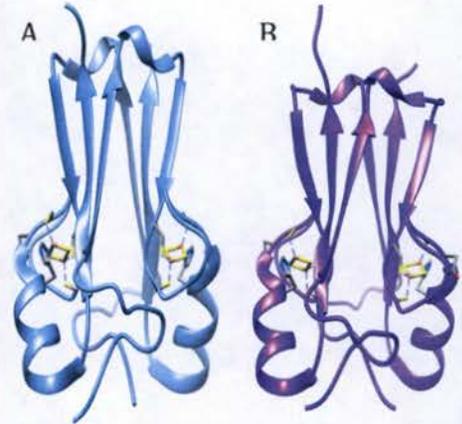


Figure 2. Crystal structure of mitoNEET (A) and Miner-1 (B). Obtained from RCSB Protein Data Bank (Structures 2QH7 and 3FNV for mitoNEET and Miner-1 respectively).

Furthermore, Miner-1 (sometimes referred to in the literature as NAF-1) is also a member of NEET family proteins and has been found to have a significant role in regulating autophagy and apoptosis^{18,19}. Increased levels of Miner-1 have been found in breast, gastric, and liver cancers. Suppressing Miner-1 expression yields reduced tumor growth, increased autophagy, and accumulation of reactive oxygen species in the mitochondria^{18,20,21}. The properties of these two unique iron-sulfur cluster proteins make them potential targets for anti-cancer and anti-diabetes drugs. Mitochondria play a key role in many human diseases because of their central functions in energy production and biosynthesis of essential cellular compounds, but how NEET family proteins integrate into these functions has yet to be fully characterized^{1,15,22}.

Uncontrolled proliferation is central to the characterization of cancer. To sustain the energy requirements for cell growth and proliferation, neoplastic tissues exhibit a metabolic adaptation in the form of a shift from oxidative phosphorylation to glycolysis as the primary energy-supplying pathway^{23,24}. This alteration in metabolism is becoming increasingly recognized as a hallmark of cancer²². Initially, this shift was attributed to the hypoxic conditions of the tumor microenvironment; however, it has been observed that this shift occurs in cancerous cells exposed to adequate concentrations of oxygen, which came to be known as the “Warburg effect”^{25,26}. Substituting galactose for glucose has been shown to ameliorate these effects in some cell lines but the mechanisms of glucose induced inhibition and galactose mediated increase in respiration are still poorly resolved²⁷. In the present study, we investigated the impact of pioglitazone, over-expression of mitoNEET and Miner-1, and galactose on human hepatocellular carcinoma (HepG2) cell bioenergetics and proliferation. HepG2 cells that were cultured in glucose or galactose based medium over 2 weeks showed strikingly lower mitoNEET levels if cultured in the presence of galactose. We hypothesize that some of the effects of pioglitazone on HepG2 bioenergetics may depend on the cellular levels of NEET family proteins, and we therefore investigated the impact of pioglitazone on HepG2 cells with low and high levels of the protein.

Materials and Methods

Chemicals

All chemicals used for respirometry and solution preparations were of the highest grade and 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 (ATCC, Manassas, VA) and grown in 75 cm² cell culture flasks (Corning Incorporated, Corning, NY). Standard cell culture medium to maintain HepG2 cells was composed of Opti-MEM I reduced serum medium (ThermoFisher, Grand Island, NY) supplemented with 5.5% fetal bovine serum (FBS) (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). Galactose treatment groups were cultured for a minimum of 4 weeks in glucose free DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10 mM D-galactose, 2 mM glutamine, 1 mM pyruvate (all from ThermoFisher, Grand Island, NY), plus 10% dialyzed or complete FBS as indicated in the figure legends (Gal-DMEM). Gal-DMEM medium prepared with complete FBS contained >0.2 mM glucose (data not shown). For experiments directly comparing the impact of high glucose and galactose, cells were cultured in the above medium supplemented with 10 mM glucose instead of galactose (Glu-DMEM). 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 subcultured every 7 days or before

reaching 90% confluency. To subculture, cells were dissociated using 0.25% trypsin and 1 mM EDTA in balanced salt solution (ThermoFisher, Grand Island, NY) and reseeded at $1.5 \cdot 10^6$ cells per 75 cm² cell culture flasks.

SDS-PAGE and Immunoblotting

Protein was isolated from 5×10^6 cells using 0.5 mL of RIPA buffer (150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 25 mM TRIS-HCl, pH 7.6) and gentle agitation for 2 h at 4 °C. Cell extracts were centrifuged for 30 min at 10,000 g and the supernatant was collected. Protein concentration in samples was determined using the Pierce™ Coomassie Plus™ (Bradford) protein assay (ThermoFisher, Grand Island, NY) with bovine serum albumin as standard. The extracts were either stored at -80°C until used, or immediately diluted 1:1 with 2X Laemmli buffer (2% SDS, 25% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue, and 62.5 mM Tris-HCl, pH 6.8). Proteins in samples were denatured at 96°C for 5 min and 15–20 μg of protein were loaded per lane on a 12% polyacrylamide gel. Gels were run using the Mini-PROTEAN 3 Cell system (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, proteins in the gel were electrophoretically transferred in buffer (192 mM glycine, 20% methanol, 0.025% SDS, and 25 mM Tris) onto a nitrocellulose membrane (0.2 μm, Bio-Rad) using the Mini Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA). Membranes were stained with Ponceau S in 0.1% glacial acetic acid to confirm transfer of proteins. The nitrocellulose membrane was then incubated in blocking buffer (5% w/v milk powder, 137 mM NaCl, 0.1% Tween-20, 20 mM Tris-HCl, pH 7.6) for 1 h. Anti-mitoNEET [2B3] mouse monoclonal antibody, anti-VDAC rabbit polyclonal antibody, and anti-β-actin rabbit polyclonal antibody (all from Abcam, Cambridge, MA) were used as primary antibodies at 1:5000 dilutions. Biotinylated protein ladder was used as molecular weight marker (Cell Signaling Technology, Danvers, MA).

The blots were incubated overnight with the primary antibody in blocking buffer at 4°C. Appropriate secondary antibodies conjugated to horseradish peroxidase were used at a dilution of 1:10,000 (Cell Signaling Technology, Danvers, MA and Abcam, Cambridge, MA). Proteins were visualized using LumiGLO (Cell Signaling Technology, Danvers, MA) and Hyperfilm ECL (GE Healthcare, Pittsburgh, PA). ImageJ 1.38e (<http://imagej.nih.gov/ij/>) was used to analyze band intensities.

Cloning of CISD1 and CISD2, Selection, and Visualization

DNA constructs (5'-CACCATGGCCCTGACTTCCTCTTCCTCTGTGCGCGTGGAGTGG ATAGCTGCTGTGACTATAGCAGCTGGAACAGCAGCCATCGGTTATTTGGCTTACAAG CGCTTCTACGTTAAGGACCACAGAAACAAGGCCATGATCAATCTCCATATCCAGAA AGACAACCCAAAGATCGTCCATGCTTTCGATATGGAGGACCTTGGAGATAAGGCTG TGTACTGCCGGTGTGGCGATCTAAGAAGTTCCCTTTTGCACGGGGCTCATACTA AACACAACGAGGAGACGGGCGATAACGTCGGCCCACTTATCATCAAGAAAAAGGA AACCGGAGGCGGCGGCAAA-3') encoding for the human mitoNEET protein (GenBank: AAH59168) and (5'-GTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAA TGCCAACTTTGTACAAAAAAGTTGGCATGGTGCTGGAGAGCGTGGCCCGTATCGTGA AGGTGCAGCTCCCTGCATATCTGAAGCGGCTCCCAGTCCCTGAAAGCATTACCGGGT TCGCTAGGCTCACAGTTTCAGAATGGCTTCGGTTATTGCCTTTCCTTGGTGTACTCGC ACTTCTTGGCTACCTTGCAGTTCGTCCATTCTCCCGAAGAAGAAACAACAGAAGGA TAGCTTGATTAATCTTAAAATACAAAAGGAAAATCCGAAAGTAGTGAATGAAATAA ACATTGAAGATTTGTGTCTTACTAAAGCAGCTTATTGTAGGTGTTGGCGTTCTAAAA CGTTTCCTGCCTGCGATGGTTCACATAATAAACACAATGAATTGACAGGAGATAATG TGGGTCCACTAATACTGAAGAAGAAAGAAGTATACCCAACTTTCTTGTACAAAGTTG

GCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAAC-3') encoding for the human Miner-1 protein (GenBank: KJ896300.1) were obtained from a commercial DNA synthesis service (IDTDNA, Coralville, IA). The synthetic DNA was amplified using the phusion high-fidelity PCR master mix (New England BioLabs, Ipswich, MA). PCR reactions were prepared by adding 0.5 μ M forward primer, 0.5 μ M reverse primer, 50 ng template DNA and PCR-grade water to the 2x master mix to yield a total reaction volume of 25 μ l. Primer sequences used were 5'-CACCATGGCCCTGACTTCCTCTTCCTCTGG-3' and 5'-TTTGCCGCCGCCTCCGGTTTC CTTTTTC-3'. Cycling parameters were 30 s at 98 °C and then 25 cycles of 98 °C (15 s) and 72 °C (60 s), followed by a final extension step at 72 °C for 10 minutes. The PCR product was cloned into the pENTR/D-TOPO cloning vector following the instructions of the manufacturer and subcloned into the pcDNATM-DEST47 destination vector using clonase technology (ThermoFisher, Grand Island, NY). The pcDNATM-DEST47 vector was chosen to express a chimeric protein composed of green fluorescence protein fused to the c-terminus of mitoNEET in HepG2 cells.

For transfections, 5 μ L of FuGENE HD (Promega, Madison, WI) was mixed with 1 μ g of plasmid DNA in 50 μ L of sterile water and incubated for 15 min at room temperature. HepG2 cells were grown in 12-well plates to a confluence of about 50% using Opti-MEM I reduced serum medium (ThermoFisher, Grand Island, NY) supplemented with 5.5% FBS without antibiotics. The reaction mixture was added to HepG2 grown in one well of a 12-well plate. The cells were transferred 24 h later to 100 cm² culture plates. After additional 24 h of incubation, geneticin (ThermoFisher, Grand Island, NY) was added to the cell culture medium at an effective concentration 800 μ g mL⁻¹. The medium was exchanged every 3 – 4 days until colonies were observed in the culture dish. Single colonies were dissociated using 0.25% trypsin plus 1 mM

EDTA in balanced salt solution (ThermoFisher, Grand Island, NY). MitoNEET-GFP and Miner-1 expressing clones were identified by fluorescence microscopy and western blotting respectively and expanded for subsequent experiments.

Respirometry on HepG2 Cells

Respiration was measured at 37 °C using 1×10^6 cells per chamber of the Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria). Routine respiration of intact cells was measured in either Opti-MEM I reduced serum medium, DMEM supplemented with glucose, or DMEM supplemented with galactose. The media formulations in these experiments were identical to the media used to culture cells. In some experiments, cellular respiration was uncoupled by successive titrations of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 0.5 μ M steps), and leak respiration was measured in the presence of oligomycin (2 μ g/mL).

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 dimethyl sulfoxide (DMSO) at 10 mg/mL (final concentration 10 μ g $\times 10^{-6}$ cells). This digitonin concentration was found to be sufficient to permeabilize the plasma membrane of HepG2 cells with minimal impact on the integrity of the outer mitochondrial membrane as tested by addition of cytochrome *c*. Electron flow through complex I was stimulated by adding 2 mM malate, 10 mM glutamate, and 5 mM pyruvate. 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 μ g/mL), and contribution of complex I to leak respiration was recorded after

addition of rotenone (0.5 μ M). Non-mitochondrial oxygen consumption was recorded after addition of 2.5 μ M of antimycin A. DATLAB software (OROBOROS Instruments, Innsbruck, Austria) was used for data analysis and acquisition.

Cell Proliferation

Approximately 250,000 cells were plated per well on 12-well plates, placed in a humidified atmosphere of 6.5% CO₂ and 93.5% air at 37 °C, and grown in glucose or galactose containing medium. After 24 h, cell counts were performed on untreated control cells. Samples for proliferation assays were exposed to pioglitazone concentrations of 0 or 60 μ M and after an additional 24 h of culture time, treated and untreated cells were enumerated using a hemocytometer. Membrane integrity was assessed by diluting samples in a 1:1 ratio with trypan blue prior to cell counts, and only trypan blue negative cells were used to calculate cellular proliferation rates. Fold increase in cell numbers were expressed as numbers of cells recovered after 24 h of plating divided by cell numbers recovered after 48 h.

Statistical Analyses

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 3-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) was used for the analyses.

Results

Respiration of HepG2

Routine respiration rates of cells cultured in medium containing 10 mM glucose or 10 mM galactose as the fuel source were significantly reduced in presence of 60 μ M pioglitazone (Fig. 3A and B). Treatment with pio reduced respiration of HepG2 cells cultured in medium containing galactose, but had no effect on those cultured in glucose-based medium. Oxygen flux of cells cultured in galactose-based medium exhibited significantly increased maximum uncoupled respiration in the presence of a carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone FCCP uncoupler. Interestingly, pio treatment appeared to have no effect on uncoupled respiratory rates in these same cells (Fig. 3B). However, drug treatment significantly

reduced maximum uncoupled respiration by 30% for HepG2 cultured in glucose-based medium (Fig. 3A). Proton leak respiration in presence of F_0 - F_1 ATPase inhibitor oligomycin ($LEAK_0$) was significantly increased in cells treated with pioglitazone. This was a common finding throughout the study.

To reveal the impacts of pioglitazone on the oxidative phosphorylation (OXPHOS) system, HepG2 cells were permeabilized, and two different substrate titration protocols

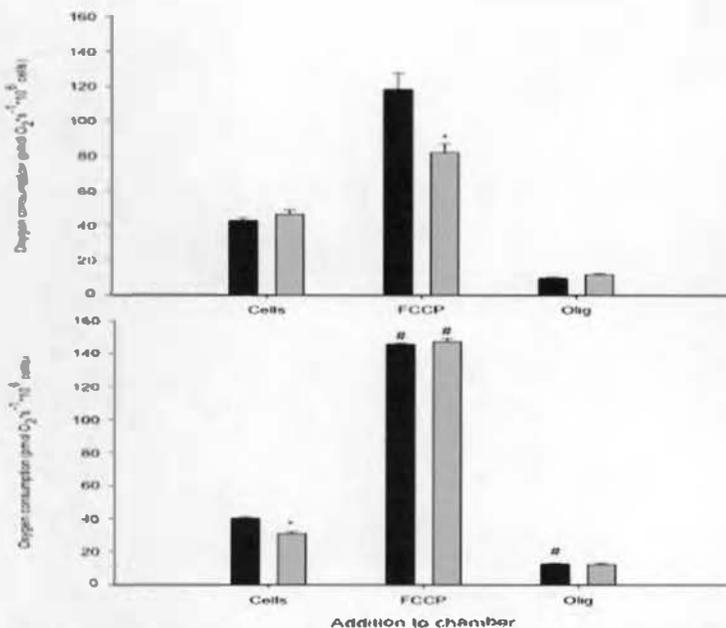


Figure 3. Respiration rates of HepG2 cells cultured in glucose (3A) or galactose (3B) based medium for two weeks. Oxygen flux is shown for control cells (black bars), and in presence of 60 μ M pioglitazone (grey bars). Maximal uncoupled respiration was induced by successive titrations with FCCP (FCCP), and leak respiration was recorded after inhibition of the F_0 - F_1 -ATPase with oligomycin (Olig). *Indicates statistically significant differences after addition of pioglitazone. #Indicates statistically significant differences between cells cultured in glucose or galactose based medium ($n = 6$, \pm SE).

were performed. The first examined complex II of the electron transport chain (ETC) in the presence of a complex I inhibitor, rotenone, with saturating levels of succinate. In the presence of succinate, pioglitazone treatment produced a significant increase in respiration by 63% and 27% for HepG2 cultured in glucose and galactose respectively (Fig. 4A and B). After engaging the phosphorylation system with ADP, drug treatment significantly lowered respiration rates for cells cultured in galactose (Fig. 4B), while there was no impact on control (Fig. 4A). Similar to previous findings, LEAK₀ respiration was significantly increased in presence of pioglitazone.

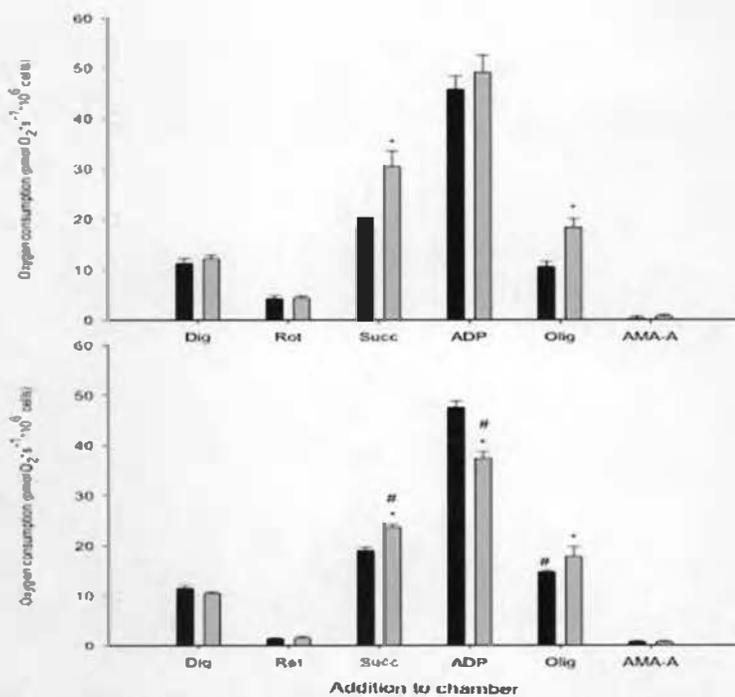


Figure 4. Respiration rates of HepG2 cells cultured in glucose (4A) or galactose (4B) based medium for two weeks. Oxygen flux is shown for control cells (black bars), and in presence of 60 μ M pioglitazone (grey bars). Respiration rates of cells permeabilized with digitonin (Dig) were recorded in presence of the Complex I inhibitor rotenone (Rot) and succinate (Succ). OXPHOS rates were measured after the addition of ADP (ADP), and leak respiration was recorded after inhibition of the F_0F_1 -ATPase with oligomycin (Olig). *Indicate statistically significant differences after addition of pioglitazone ($n = 6, \pm$ SE).

The second protocol examined the impact of treatment on complex I in the presence of both NADH and FADH₂ generating substrates for HepG2, HepG2 that were engineered to over-express the protein mitoNEET (HepG2-NEET), HepG2, and Miner-1 (HepG2-Miner). Overexpression of mitoNEET resulted in significant increases in ADP and succinate-stimulated respiration as well as an increase in LEAK₀ rates (Fig. 5). Similarly, respiration rates of HepG2-Miner cells after the addition of

succinate in presence of ADP were increased (Fig. 5). Drug treatment severely inhibited complex I of both HepG2 and HepG2-NEET cells and increased LEAK₀ respiration rates (Fig. 5). This was evident from the large reduction of oxygen flux after the addition of ADP. Culturing all

three cell lines in galactose resulted in significant increases in oxygen flux after engaging the phosphorylation system with ADP (Figs. 5 and 6). Additionally, galactose-based medium increased LEAK₀ respiration for all three-cell lines, while HepG2-Miner cells exhibited the largest increase in proton leak (Figs. 5 and 6).

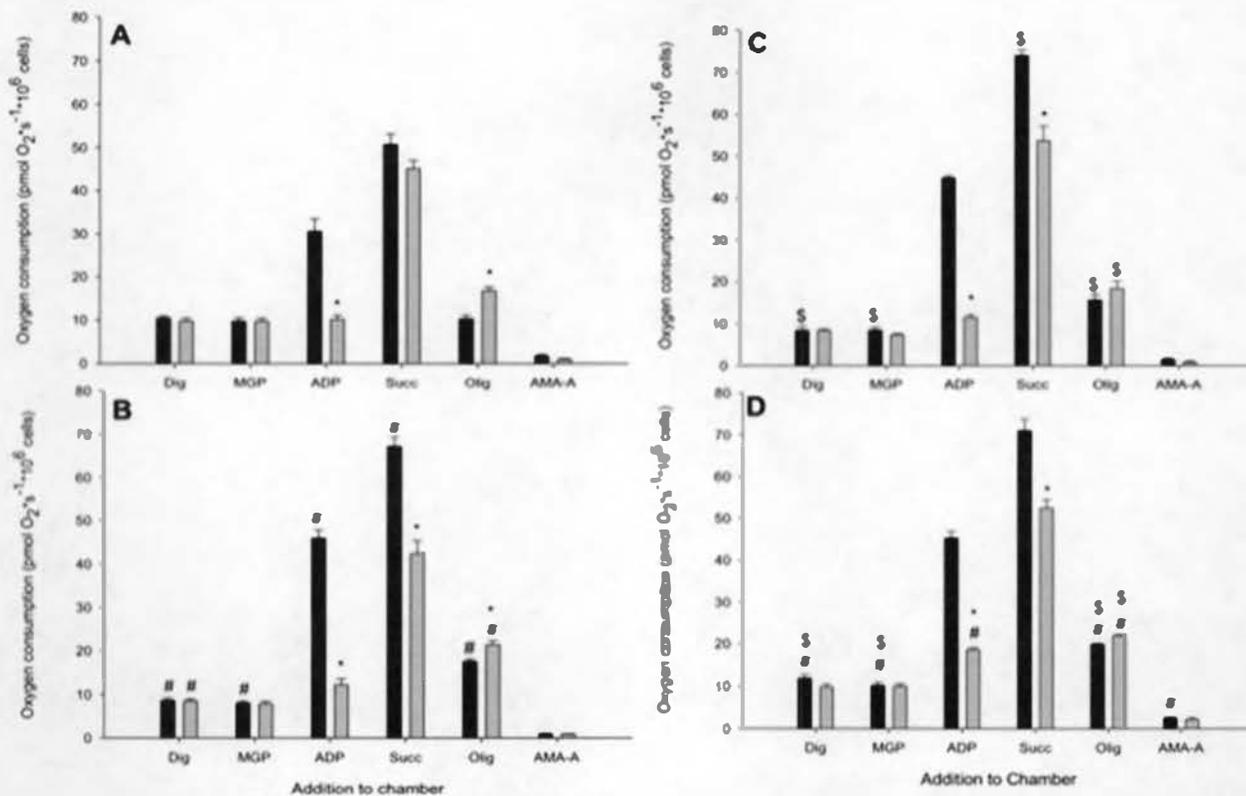


Figure 5. Respiration rates of HepG2 (5A and B) or HepG2 engineered to over-express the protein C1SD1 (HepG2-NEET, 5C and D) cells cultured in glucose (5A and C) or galactose (5B and D) based medium for two weeks. Oxygen flux is shown for control cells (black bars), and in presence of 60 μM pioglitazone (grey bars). Respiration rates of cells permeabilized with digitonin (Dig) were recorded in presence NADH generating substrates (MGP) and OXPHOS rates were measured after the addition of ADP (ADP). Convergent electron entry into the ubiquinone pool was initiated by addition of succinate (Succ) and leak respiration was recorded after inhibition of the F₀F₁-ATPase with oligomycin (Olig). *Indicate statistically significant differences after addition of pioglitazone. #Indicate statistically significant differences between cells cultured in glucose or galactose containing medium. §Indicate statistically significant differences between cell lines (*n* = 6, ± SE).

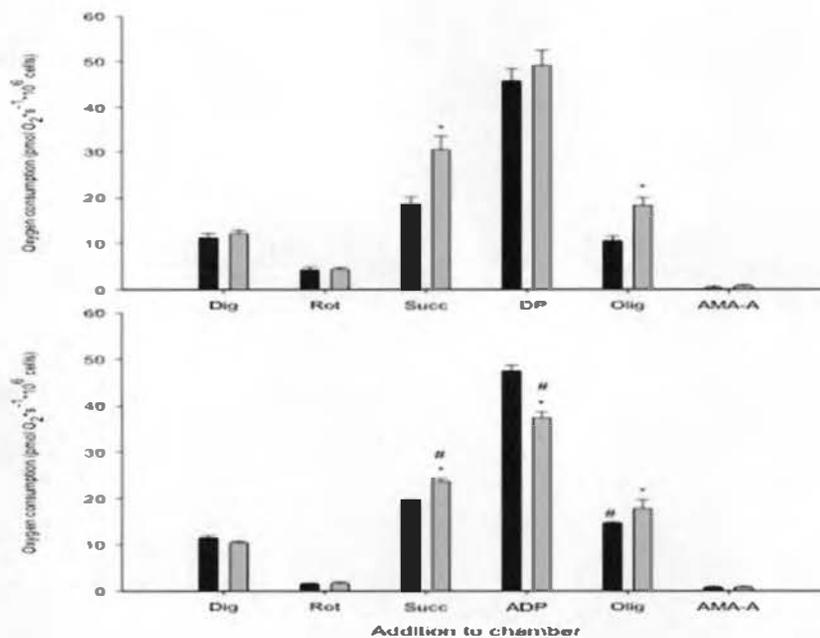


Figure 6. Respiration rates of HepG2 cells that were engineered to over-express the protein C1SD2 (HepG2-2) cultured in glucose (Black Bars) or galactose (Gray Bars) based medium for two weeks. Respiration rates of cells permeabilized with digitonin (Dig) were recorded in presence NADH generating substrates (MGP) and OXPPOS rates were measured after the addition of ADP (ADP). Convergent electron entry into the ubiquinone pool was initiated by addition of succinate (Succ) and leak respiration was recorded after inhibition of the F₀F₁-ATPase with oligomycin (Olig). #Indicate statistically significant differences between cells cultured in glucose or galactose containing medium ($n = 2$ and 4 for glucose and galactose, respectively, \pm SE).

Western Blot Analysis and Cell Proliferation

Cell culture medium containing 10 mM galactose significantly reduced cellular levels of mitoNEET compared to those cultured in 10 mM glucose (Fig. 7). However, levels of the housekeeper β -actin remained similar, regardless of the culture medium used. When cultured with a combination of glucose and galactose, less reduction of mitoNEET was observed (Fig. 7B and D). Levels of the mitochondrial housekeeper VDAC remained the same despite changes in medium composition. Proliferation of HepG2 cultured in galactose-based medium was significantly reduced compared to glucose controls (Table 1). Treatment with pioglitazone reduced the fold-increase of cells cultured in glucose, but the same treatment failed to produce a difference in HepG2 proliferation after being cultured in galactose for 2 weeks.

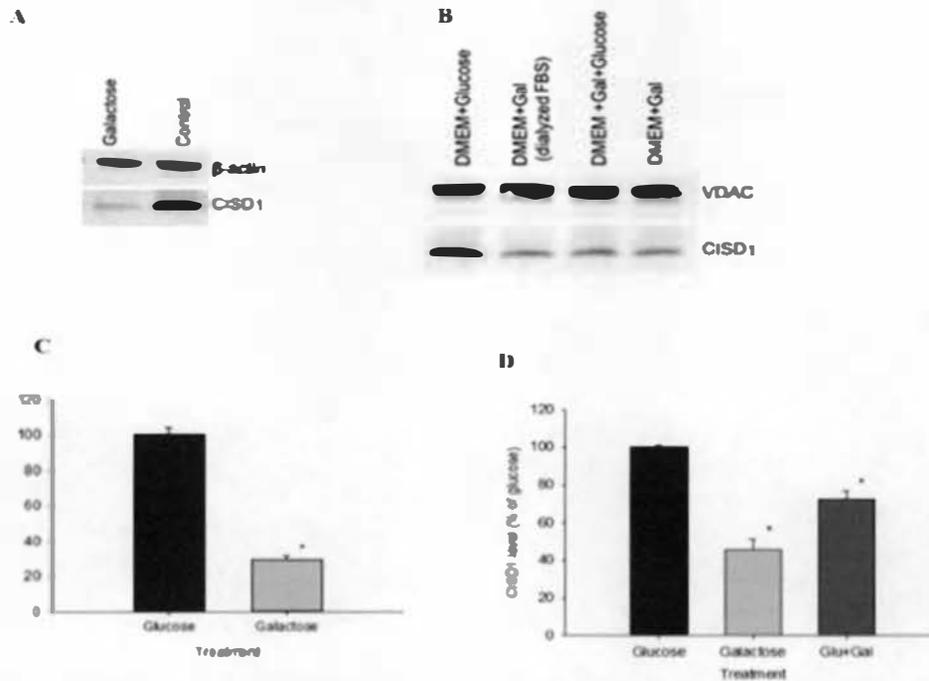


Figure 7. Medium supplemented with D-galactose significantly reduces cellular Cisd1 levels compared to high glucose controls in HepG2 cells. Protein levels were measured by immunoblotting. A) Cisd1 levels are reduced in galactose medium compared to control; β -actin was used as cellular housekeeper protein. B) Cisd1 levels are decreased in medium supplemented with galactose (Gal) or both galactose and glucose, VDAC served as mitochondrial housekeeper. C) Band intensities were quantified using the software ImageJ, Cisd1 bands were normalized based on the intensity of the housekeeper proteins and expressed as percentage of glucose controls ($p < 0.05$, $n = 3$, \pm SEM).

Table 1. Impact of galactose and pioglitazone treatment on proliferation rates of HepG2 cells. *Indicate statistically significant differences after treatment with 60 μ M pioglitazone. #Indicate statistically significant differences in proliferation between cells cultured in glucose and galactose.

Condition ¹	Fold-increase in cells
Glu (Vc)	2.19 \pm 0.15
Glu (Pio)	1.77* \pm 0.02
Gal (Vc)	1.37# \pm 0.05
Gal (Pio)	1.17# \pm 0.15

Discussion

Our study investigated the impact of pioglitazone as well as over-expression of mitoNEET and Miner-1 on HepG2 bioenergetics after shifting metabolic activity from glycolysis to oxidative phosphorylation by using a cell culture medium that contained galactose instead of glucose. Maximum uncoupled respiration rates were reduced in the presence of 60 μ M pioglitazone for cells cultured in 10 mM glucose, but not for those cultured in 10 mM galactose. Pioglitazone harshly reduced activity of complex I in permeabilized cells, but this effect appeared to be mitigated by addition of succinate to supply FADH₂ to complex II for subsequent electron delivery to the ubiquinone/ubiquinol pool. Moreover, using a galactose-based cell culture medium greatly reduced endogenous levels of mitoNEET and significantly slowed proliferation of HepG2. Additionally, pioglitazone was found to reduce proliferation in cells cultured in both glucose and galactose-based medium. These results increase our understanding of the molecular actions of pioglitazone and suggest that galactose may be used as a novel chemical tool to reduce cellular levels of mitoNEET.

Pioglitazone has been found to change mitochondrial energetics through multiple mechanisms. Complex I-fueled respiration rates of primary hepatocytes were reduced in response to treatment with pioglitazone⁹. Furthermore, examination of isolated mouse liver mitochondria revealed that pioglitazone treatment reduced complex I and III activity, as well as disassembled complex I into four discrete subcomplexes². Moreover, pioglitazone has been found to inhibit pyruvate-driven oxygen consumption and decrease glucose production in liver cells^{2,28}. Energetically speaking, glycolysis is an inefficient method of ATP production for cellular processes. However, highly proliferative cells have greater carbon and nitrogen requirements to maintain their growth. Glucose and glutamine are the two most utilized

compounds for energy, carbon, and nitrogen requirements for proliferation in mammalian cell culture^{29,30}. Catabolism of galactose yields no net ATP, thus it is hypothesized that culturing cells in galactose forces energy yield through oxidative phosphorylation²⁷. Our respiration and proliferation results confirm this hypothesis, but future works elucidating the utilization of glutamine to fuel OXPHOS through its conversion to α -ketoglutarate in the presence of galactose as the principal sugar molecule are required to better determine the role of galactose in energy production in immortalized cell lines.

Additionally, mitochondrial metabolism is becoming recognized as a new therapeutic target for anti-cancer drugs due to the discoveries surrounding tumor promotion and mitochondrial biogenesis^{18,31}. MitoNEET has been implicated in stimulating mitochondrial biogenesis and resisting autophagy in cancer cells³¹. Cells with lower levels of mitoNEET have been shown to exhibit reduced oxidative capacity and accumulation of iron and reactive oxygen species (ROS) in the mitochondrion. In several breast cancer cell lines, levels of mitoNEET were significantly increased, which corresponded with an increase in mitochondrial respiratory complexes I, III, IV, and V and a decrease in autophagy^{31,32}. Interestingly, overexpression of mitoNEET led to increased tumor growth independent of angiogenesis while mitoNEET deficiency conferred significant tumor mass reduction.

Furthermore, exposure to galactose decreased endogenous levels of mitoNEET. Culturing cells in galactose-based medium also reduced levels of the transgenic-expressed mitoNEET-GFP under the control of human cytomegalovirus promoter. Given the differences in the promoters and sequences between the endogenous and synthetic protein, we postulate that galactose likely increases proteasomal degradation of mitoNEET. Whether galactose targets mitoNEET directly or disrupts the iron-sulfur cluster assembly is not known. However, a recent study found that

interference with cluster assembly marks mitoNEET for proteasomal degradation³³. In light of this finding, future metabolic studies performed on immortalized cell lines cultured in galactose-based medium should be thoroughly evaluated as galactose induces complex changes to the mitochondrial proteome.

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