THE EFFECT OF ISOLEUCINE SUPPLEMENTATION ON PERIPHERAL BLOOD MONONUCLEAR CELL METABOLISM IN SUBJECTS WITH TYPE 2 DIABETES

An Undergraduate Research Scholars Thesis

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ABSTRACT

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Type 2 diabetes (T2D) is an increasing health concern as 425 million people are diagnosed with it worldwide. It is associated with chronic systemic inflammation and increased oxidative stress; individuals with these inflammatory conditions have altered metabolic pathways and mitochondrial function including changes in mitochondrial respiration values in peripheral blood mononuclear cells (PBMCs). Mitochondrial respiratory capacity is vital to produce cellular energy in the form of adenosine triphosphate (ATP) via the tricarboxylic acid (TCA) cycle and mitochondrial electron transport chain (ETC). Metabolic dysfunction associated with systemic inflammation can lead to limited production of tricarboxylic acid (TCA) cycle intermediates which are vital for metabolism. It has previously been shown that providing anaplerotic TCA cycle precursors, specifically isoleucine and valine, can replenish TCA cycle intermediates. The purpose of this study was to assess the mitochondrial function in PBMCs from eight control subjects and seven T2D subjects using high resolution respirometry. Subjects with T2D received ten days of treatment with three grams daily of supplemental isoleucine. PBMC respiration was compared between control and T2D at baseline, and T2D subjects were

assessed for changes over the treatment duration. Subjects with T2D had significantly lower leak respiration and leak-related coupling control ratio than healthy control subjects. There was no significant change in measures of PBMC respiration from T2D subjects before and after treatment. Although there appears to be minor differences in PBMC respiratory rate between subjects with T2D and healthy controls, 10 days of isoleucine supplementation was not effective at recovering altered PBMC respiration in T2D subjects.

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NOMENCLATURE

T2D	Type 2 Diabetes
PBMC	Peripheral Blood Mononuclear Cells
ATP	Adenosine triphosphate
TCA	Tricarboxylic acid
ETC	Electron transport chain
BCAA	Branched chain amino acid
ONS	Oral nutritional supplement

CHAPTER I

INTRODUCTION

T2D Background

Type 2 diabetes (T2D) is a leading health concern in the United States, affecting about 9.4% of Americans (*Division of Diabetes Translation At A Glance | CDC*, 2019). T2D is a metabolic disorder resulting in hyperinsulinemia and hyperglycemia and characterized by insulin resistance in tissues including liver, skeletal muscle, and adipocytes (Hameed et al. 2015). If left unmanaged, diabetics are at greater risk of other health problems including nonalcoholic fatty liver disease, kidney disease, and cardiovascular disease (Piette and Kerr 2006). The primary strategy to manage T2D is to incorporate healthy eating habits in conjunction with physical activity to lower blood sugar levels to reverse the development of chronic complications associated with diabetes. In addition to diet and exercise, pharmacological interventions include antidiabetic medication and insulin therapy (Polonsky and Henry 2016). Examples of medications include those that work to lower blood sugar, often by improving cell sensitivity to insulin (Polonsky and Henry 2016). With proper management, subjects with T2D can maintain blood glucose within a normal range (Polonsky and Henry 2016). Because so many Americans are affected by T2D, research into alternative strategies to manage diabetes has the potential to have a large impact.

Metabolic Mechanisms

Metabolism is the overarching process of utilizing food and nutrient intake to be converted into energy and encompasses all catabolic and anabolic reactions in the body (Mailloux et al. 2007). Metabolic pathways are often controlled by activating or inhibiting

regulatory enzymes to determine the reaction rate. The activation or inactivation of certain enzymes is also regulated via compartmentalization, meaning that certain reactions occur in specific organelles (Metallo and Vander Heiden 2013). Metabolic pathways regulating the conversion of chemical energy into useful energy are regulated via a series of intermediate reactions (Metallo and Vander Heiden 2013). The energy used to fuel these pathways is generated by the metabolic breakdown of carbohydrates, lipids, and proteins (Hardie 2012). Metabolic breakdown of these fuels for energy converges at the tricarboxylic acid (TCA) cycle within the mitochondria (Mailloux et al. 2007). The TCA cycle converges with the electron transport chain (ETC) to generate energy in the form of adenosine triphosphate (ATP) (Mailloux et al. 2007). Ultimately the body's physiological processes are concerned with maintaining homeostasis and ensuring that the body has enough energy to function effectively. Metabolic mechanisms carry this out and demonstrate the requirement of properly functioning metabolism.

Mitochondria's Role in Energy Capacity

In previous studies it has been determined that insulin resistance is associated with mitochondrial dysfunction in muscle tissue (Larsen et al., 2009). In muscle tissue, it has been demonstrated that the total volume of the mitochondria in the muscle is directly proportional to the oxidative capacity. Mitochondrial dysfunction has been linked inherited human disorders and is common in diseases such as cancer, obesity, and neurodegenerative disorders (Nunnari & Suomalainen, 2012).

Mitochondria are present in almost all eukaryotic cells and are responsible for a variety of important cellular processes such as energy production, calcium homeostasis, phospholipid synthesis, cell cycle control, and cell death (Osellame et al. 2012). The coordination of the necessary intermediaries allow for proper functioning of the mitochondrial respiratory chain.

The main driver in aerobic mitochondrial metabolism is the proton gradient (Schroeder et al. 2009). The TCA cycle makes NADH and $FADH_2$, which carry electrons to the inner mitochondrial membrane (Martinez-Reyes and Chandel 2020). These carriers release the protons and electrons to a series of proteins on the inner mitochondrial membrane that utilize the energy from the transfer of electrons to drive the proton motive force in the ETC (Martinex-Reyes and Chandel 2020). After arrival at the inner mitochondrial membrane, the electrons are accepted by ETC protein complexes I and II and are used to pump protons into the intermembrane space, creating a potential difference between the matrix and intermembrane space (Martinex-Reyes and Chandel 2020). This proton gradient is what ultimately powers the generation of the majority of aerobic ATP production. The proton gradient favoring flux of proteins back to the interior of the matrix is used to power ATP synthesis (Schroeder et al. 2009).

The proper functioning of the TCA cycle is important for both energy production as well as for biosynthesis of precursors for other metabolic pathways. If TCA cycle intermediates are removed as precursors for biosynthesis, the cycle is not able to function unless the intermediates are replenished by anaplerosis. Depletion of metabolic intermediates can result in disruption of metabolism and oxidative stress due in part by leakage of electrons directly to oxygen subverting the ETC. This oxidative stress can eventually lead to cellular damage and necrotic cell death (Zhao et al. 2019). As the primary driver of aerobic metabolism, mitochondria are of vital importance to maintaining cellular homeostasis, energy production, and biosynthetic needs.

Inflammation and Metabolism in T2D

T2D is characterized by inflammation, hyperglycemia, insulin resistance, as well as other factors (van Greevenbroek et al. 2013). The inflammatory markers associated with T2D are seen by pro-inflammatory cytokine production and amplified by adipokines (Tsalamandris et al.,

2019). The immune system is activated with the incidence of T2D in adipose tissue inflammation (Tsalamandris et al., 2019). Ongoing inflammation resulting from an immune response leads to changes in metabolic activity (Kominsky et al., 2010). These changes in metabolic activity include depletion of nutrients, increased oxygen consumption, and increased production of nitrogen intermediates (Kominsky et al., 2010). All of the aforementioned results occur due to the recruitment of inflammatory cells, primarily monocytes and neutrophils (Kominsky et al., 2010). The action of monocytes is part of the adaptive immune response when the cells are recruited to inflammation sites (Kominsky et al., 2010).

The corresponding cell migration to the inflammatory site requires energy. At the site of inflammation, the nutrient, energy, and oxygen demands are increased in order to carry out these actions (Kominsky et al., 2010). Sites with chronic immune responses and inflammation become depleted of their nutrients, energy, and oxygen, paralleling physiological hypoxia and decreased metabolic efficiency (Kominsky et al., 2010). Insulin resistance occurs with lessened insulin sensitivity in target cells (van Greevenbroek et al., 2013). Insulin resistance parallels decreased metabolic control and increased lipid and glucose concentration. In both the liver and muscle, this ultimately results in insulin resistance (Muoio & Newgard, 2008). In the muscle, this means that lipid-derived intermediates will accumulate in the mitochondria to yield both mitochondrial stress and insulin resistance (Muoio & Newgard, 2008).

Cellular stress occurs physiologically in response to a stressor (Mechanik, 2006). The TCA's cycle limited ability to compensate for this cellular stress leads to decreased oxidative phosphorylation capacity and less ETC activity, characteristic of mitochondrial insufficiencies. The overall stress response in subjects with T2D diverts metabolic fuel from anabolic functions. To provide for the inflammatory response, tissue catabolism increases to provide the amino acids

necessary for protein synthesis. These pro-inflammatory effects of hyperglycemia are related to reactive oxygen species (ROS) and cytokines specifically (Mechanick, 2006). As this discussion documents, T2D negatively impacts the function of metabolic mechanisms by having to prioritize the most necessary physiological functions for the body.

Mitochondria and This Study

Mitochondrial respiratory capacity is a measure of mitochondrial oxygen consumption to produce cellular energy in the form of ATP via the TCA cycle and mitochondrial ETC. The TCA cycle is necessary for energy production and aerobic respiration (Schroeder et al. 2009). Metabolic dysfunction associated with systemic inflammation can lead to limited production of TCA cycle intermediates which are vital for metabolism (Green et al. 2016). It has previously been shown that providing TCA cycle precursors, specifically isoleucine and valine, can replenish succinyl Co-A in the TCA cycle (Green et al. 2016). The branched-chain amino acids (BCAAs) consist of isoleucine, valine, and leucine. The BCAA isoleucine was used in this study because evidence from non-obese diabetic mice has consistently demonstrated that aromatic amino acids and BCAAs are two of the most significantly impacted pathways to improve inflammatory symptoms (Liu et al. 2017). BCAAs are used rather than aromatic amino acids because supplementation of BCAAs is thought to promote anabolic pathways (Holeček 2018).

This research assessed metabolism in peripheral blood mononuclear cells (PBMCs) in subjects with T2D and age matched controls. Individuals with these inflammatory conditions have altered metabolic pathways and mitochondrial function including changes in respiration values of PBMCs (Roe and Mochel 2006). The objective of this study was to determine whether an isoleucine supplement would promote the TCA cycle to combat insulin resistance and the metabolic mechanisms responsible for this disparity. In the current study we assessed

mitochondrial respiration in PBMCs of subjects with T2D using an Oroboros O2k (Oroboros Instruments; Innsbruck, Austria) for high resolution respirometry. PBMCs offer a minimally invasive way to measure cellular respiration using a simple blood draw (Hedges et al. 2019). In this study, subjects with T2D consumed an isoleucine supplement and were assessed at baseline and again immediately following 10 days of treatment. This served to assess the role of an isoleucine supplement in alleviating inflammation and oxidative cellular stress.

This research could lead to a novel and potentially effective way to provide a nutritional intervention for subjects with T2D. The efficacy of the cell's current activity and mitochondrial function is directly correlated to its ability to produce energy in the form of ATP. Because of this, treatment may potentially serve to improve the function of their mitochondria, thereby providing for increased ETC functionality, and leading to increased oxidative capacity.

Benefits of a Nutritional Intervention

A plethora of medications are available for and administered to subjects with T2D; in conjunction with these, subjects are typically also given advice on how to alter their diet. An oral nutritional supplement (ONS) is a supplementation to normal dietary food specifically for medical purposes, and ONS in conjunction with dietary advice have been shown to be more effective than dietary advice alone (Baldwin and Weekes 2011). Whereas typical T2D medications address specific symptoms of diabetes such as blood pressure and blood sugar, a nutritional intervention affects the subject's metabolism at the cellular level. By administering a supplement to the subject rather than simply giving dietary advice, one can have increased assurance that the treatment is implemented. Amino acids in particular allow for the body to obtain the levels it needs to continue proper functioning (Odia & Esezobor, 2017). The ultimate

goal of the study was to facilitate an effective and implementable intervention for subjects with T2D.

Isoleucine is classified as an essential amino acid, meaning that it must be obtained by the diet because the body is unable to synthesize it (Holeček 2018). In addition, isoleucine is a hydrophobic amino acid, meaning that it can pass through the cell's plasma membrane and enter the cell, allowing it to affect greater change (Brosnan and Brosnan 2006). It has previously been suggested that supplementation of BCAAs may help to improve the repair of damaged muscle (Monirujjaman and Ferdouse 2014). Amino acids, including isoleucine, are classified as hydrosylates, meaning that they exhibit antioxidant properties (Dash and Gosh 2017). This is vital in combatting the oxidative stress that can result in subjects with T2D (Dash and Gosh 2017). Importantly, BCAAs promote muscle cell metabolism and glucose uptake by the liver, giving them the potential to decrease the high blood glucose present in T2D subjects (Monirujjaman and Ferdouse 2014).

Peripheral Blood Mononuclear Cells

PBMCs are classified as a peripheral blood cell with a round nucleus (Navas et al. 2019). Examples of these include lymphocytes and monocytes, which are involved in the immune system and immune response (Navas et al. 2019). PBMC respiration is a marker of inflammatory conditions (Kleiveland, 2018). The subject's physiological status includes hormone levels, nutritional status, and inflammation, which affect the PBMC composition (Kleiveland, 2018).

Study Overview

The BCAA administered to subjects with T2D in this study was isoleucine. Blood was collected and PBMCs isolated from control and T2D subjects to measure mitochondrial function.

PBMCs were separated from whole blood using centrifugal separation with density gradient media. Cell counts were done using a hemocytometer to provide a means for determining relative oxygen consumption. Using an Oroboros O2K oxygraph, substrates, uncouplers, and inhibitors were added by stepwise titration into the oxygraphy resolution respirometry chamber to determine PBMC respiration at various respiratory states. These respiratory states include leak respiratory capacity, maximum coupled oxidative phosphorylation (OXPHOS), and uncoupled maximal electron transport system respiratory capacity. Detailed analysis of the high resolution respirometry results was useful to show how isoleucine supplementation affected PBMC cellular respiration in T2D. The knowledge gathered from the effect of isoleucine on T2D subjects will serve as a basis for further nutritional and supplemental interventions for T2D and other inflammatory conditions.

CHAPTER II

METHODS

Study Inclusion Criteria

To be included in this study, subjects had to be between 45 and 84 years of age with a body mass index (BMI) greater than or equal to 28 kg/ m^2, a stable body weight that maintained a range within five percent of their baseline weight over the last three months, and a physical exam, medical history, and lab screening that indicated that the subject was of suitable health. The subject had to be able to independently walk, stand, and sit down, lie down for up to 10 hours at a time, and was able and willing to follow the experiment protocol. T2D subjects had to be clinically diagnosed and currently taking insulin or an oral medication to lower their glucose. Data from seven subjects with T2D and eight healthy control subjects are included in this study.

Treatment

T2D, characterized by insulin resistance, is associated with chronic systemic inflammation and increased oxidative stress, ultimately leading to mitochondrial dysfunction (Vikram et al. 2014). In the current study we assessed mitochondrial respiration in PBMCs of subjects with T2D using an Oroboros O2k (Oroboros Instruments; Innsbruck, Austria) for high resolution respirometry. PBMCs offered a minimally invasive way to measure cellular respiration using a simple blood draw.

In this study, subjects with T2D consumed a supplement of three g isoleucine a day for 10 days. Subjects were assessed at baseline and again immediately following 10 days of treatment. This study assessed the efficacy of a simple isoleucine nutritional supplement to alleviate cellular stress, inflammation, and metabolic disturbances resulting from mitochondrial

dysfunction associated with T2D. In addition to samples being collected from subjects with T2D, blood samples were also collected from control subjects without T2D to allow for the differences in their mitochondrial oxygen consumption to be analyzed. The age matched healthy control subjects had only baseline experimental data because they were not administered a treatment.

PBMC Isolation

Blood was collected from subjects with T2D and PBMCs were isolated using centrifugal separation with density gradient media. This was done by first mixing the 10 mL blood sample with 10 mL of phosphate-buffered saline (PBS) (BIORAD, 1610780) and layering this dilution on top of 15 mL Ficoll (GE Healthcare). Ficoll is a highly branched high-mass hydrophilic polysaccharide that dissolves readily in aqueous solutions. This was centrifuged at 1000 G for 30 minutes with no acceleration or deceleration to yield density-based separation (Fig. 1). The cloudy PBMC layer was transferred to a new tube and diluted with approximately 7 mL of PBS, then centrifuged at 250 G for 10 minutes at 20 °C. The resulting cellular pellet was then resuspended in 5 mL Mir05 and 2.4 mL of the sample was transferred to each O2K chamber for high resolution respirometry. 100 µL was sub-sampled from each chamber into a tube with 400 µL of .4% trypan blue and inverted to mix. This was used for cell counts after the mixture had 5-15 minutes to stain the cells.

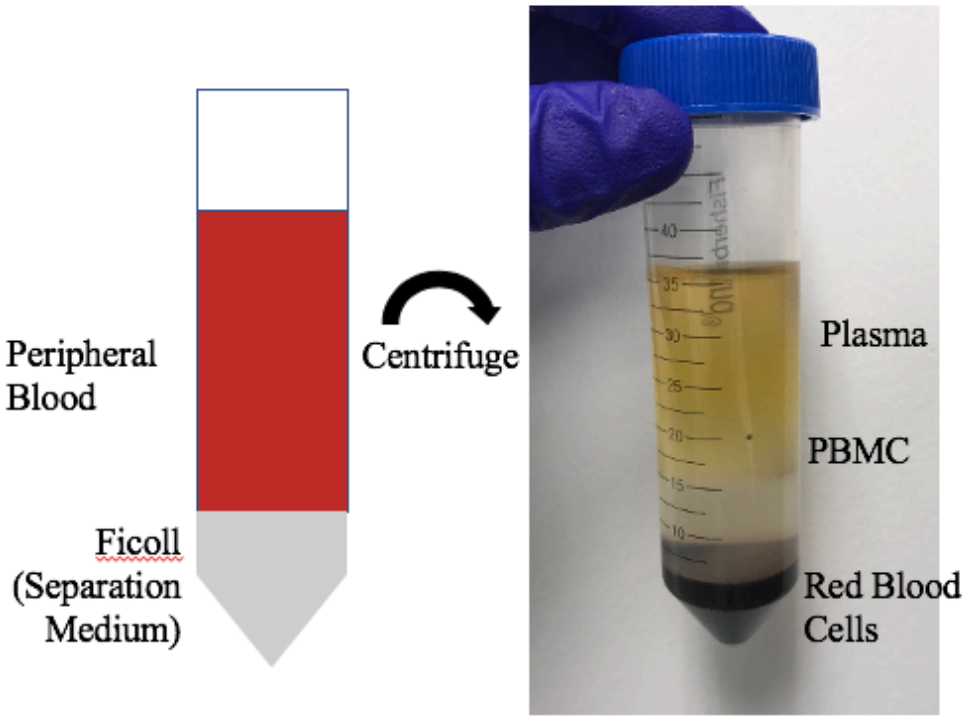

Figure 1: PBMC Isolation. Seperation of the different parts of blood using centrifugal separation. The left image shows the top red layer consisting of blood mixed with PBS carefully layered above the Ficoll. The right image is the result after centrifuging, when the different layers separate based on their relative densities.

Cell Count

Cell counts were done using a hemocytometer to provide a means for determining relative oxygen consumption. 12 µL of the above cell mixture was pipetted into one side of the hemocytometer grid and counted using a microscope. Cell density in million cells/ mL was calculated by averaging the viable cell count from each of the four grids and multiplied by 50,000 to correct the volume of 1/10,000 mL per grid and the 1:5 stain dilution.

High Resolution Respirometry

High Resolution Respirometry was performed using an Oxygraph-2K (O2K) respirometer (Oroboros Instruments, Innsbruck, Austria), and data was collected by a computer using DatLab software (Oroboros Instruments, Innsbruck, Austria). Before the substrate,

uncoupler, and inhibitor (SUIT) protocol began, the oxygen electrode was calibrated. After oxygen calibration, the cell samples were inputted into the chamber and the stopper was used to partially seal the chamber. Oxygen was injected into the chamber and the oxygen concentration was tracked so that once the oxygen buffer concentration reached 400 nmol/ml, the chamber was fully sealed to force the remaining gas out. Throughout the protocol, oxygen concentration was maintained between 250 and 400 nmol/ml. Once the oxygen concentration stabilized, respiratory flux baseline was established in the absence of metabolic precursors. The SUIT protocol, stepwise titration of substrates, adenylates, and inhibitors was used to establish specific respiratory states (Table 1). For each metabolic state, a new steady state was established before the titrations for the next metabolic state could be performed.

Table 1: SUIT protocol was administered with stepwise titration of substrates, adenylates, and inhibitors to achieve the following PBMC respiratory states: permeabolized, leak, OXPHOS, Electron Transport System (ETS), and complexes I, II, and IV. This table demonstrates the cellular state each substrate, adenylate, and inhibitor was administered to facilitate.

Respiratory State	Solution
Routine	PBMCs in MiR05
Permeabolized	Digitonin
Leak	Pyruvate
	Malate
CI	ADP
OXPHOS	Glutamate
	Succinate
ETS	CCCP
CII	Rotenone
No respiration	Antimycin A
CIV	Ascorbate
	TMPD
Inhibit CIV	Azide

The reagent concentrations used in this SUIT protocol were: 8.1 µM digitonin, 5 mM pyruvate, 1 mM malate, 1.75 mM ADP, 5 mM glutamate, 10 mM succinate, 0.5 µM CCCP,1.5 µM rotenone, 1.875 µM antimycin A, 2 mM ascorbate, 0.3 mM TMPD, and 10 mM azide. The respiratory states initiated by these protocols include: routine, permeabolized, leak, OXPHOS, and ETS.

Routine Respiration represents the untreated baseline respiratory rate, and is initiated by equilibrating PBMCs in MIRO5 buffer to temperature and determining respiratory rate in unaltered conditions.

Permeabolized respiratory capacity was then induced by titrating the permeabolizing agent digitonin resulting in washing substrates and adenylates out of the cell. This respiratory state sets the stage for stepwise reintroduction of specific substrates.

Leak respiration was induced by reintroducing the substrates pyruvate and malate for electron influx through complex I, but without adenylates to allow ATP synthase activity to determine maximum uncoupled respiratory capacity.

CI respiration was induced with addition of the adenylate ADP to allow ATP synthase activity and maximize the respiratory flux through complex I.

OXPHOS respiration was induced with addition of glutamate and succinate to provide substrates for complex II. OXPHOS represents the cell operating at maximum respiratory capacity with mitochondrial oxidative phosphorylation coupled with ATP synthase and native uncoupled leak respiration.

Electron Transport System (ETS) respiratory rate was induced with the uncoupler CCCP to artificially elevate the proton leak and determine the cell's oxidative capacity beyond OXPHOS.

CII respiration was induced with addition of rotenone, a complex I inhibitor, to represent the maximum respiratory capacity driven by complex II.

No respiration was induced by the inhibitor antimycin, because it represses cellular respiration. This gives the baseline respiratory flux that the other values are corrected for.

CIV was induced with ascorbate and TMPD. These reducing agents subvert the TCA cycle and provide direct maximal electron transfer to maximize ETS flux. Complex IV was then inhibited with azide, and the difference between these two values provides the flux contribution of complex IV.

These respiratory states are shown for the described high resolution respirometry SUIT protocol (Fig. 2). In addition to each of the steady state respiratory rates, respiratory ratios were calculated to determine relative respiratory capacity for various respiratory states. To assess complexes I and II's respective contributions to total OXPHOS capacity, the substrate control ratio (SCR) was determined: SCR_{CI} = CI / OXPHOS and SCR_{CII} = CII / OXPHOS. To determine the relative contribution of leak respiration to total OXPHOS respiration, the coupling control ratio was calculated (CCR = LEAK / OXPHOS). Finally, the respiratory control ratio was calculated to characterize the mitochondrial ETS respiratory reserved capacity that exceeds the OXPHOS capacity (RCR = OXPHOS / ETS).

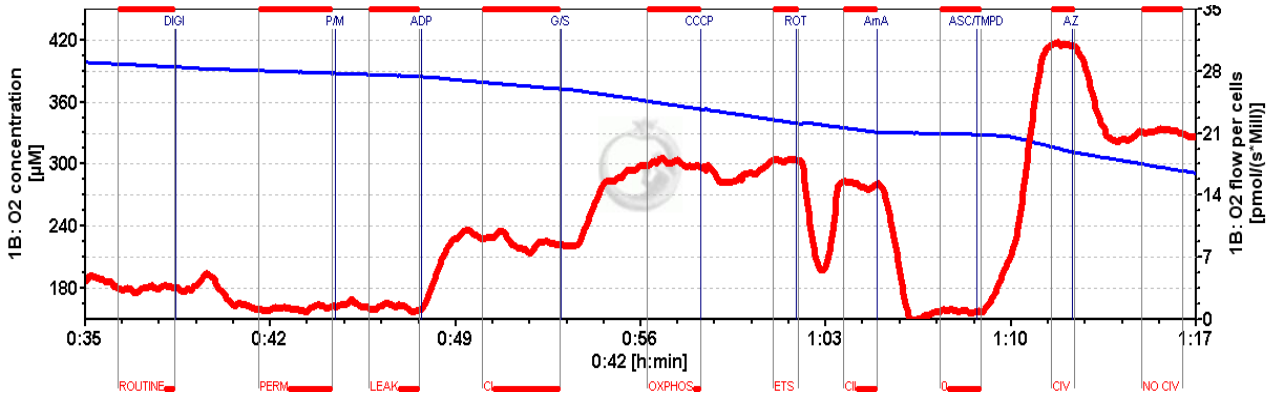

Figure 2: High resolution respirometry. Example run after employing the SUIT protocol shows the oxygen consumption and oxygen flow in each of the cellular states.

Data Analysis

Data was analyzed using GraphPad Prism 5 (Graph Pad Software, Inc., San Diego, CA) and expressed as the mean and standard deviation. Comparisons between the healthy control and pre-treatment T2D was measured using a t-test with a significance threshold of $p < 0.05$. Comparisons between pre and post-treatment measures were assessed using paired t-tests with a significance threshold of $p < 0.05$. Due to requirements limiting processing time, each sample was run redundantly in two separate chambers. Chamber A was used for analysis by default unless there was an insufficient cell count, operator error, or hardware error that required alternative use of chamber B.

CHAPTER III

RESULTS

We measured PBMC respiratory rates in various respiratory states for healthy control subjects and subjects with T2D both before and after treatment. The initial measure of routine respiration represented the baseline respiratory rate of unaltered PBMC cells introduced into respiratory buffer. After permeabolization, the respiratory rate decreased to low levels due to the washing out of substrates and adenylates in preparation of stepwise reintroduction of substrates and adenylates (Fig. 3). Reintroduction of substrates to induce leak respiration resulted in negligible increase in respiration, indicating low levels of PBMC leak respiration. The addition of the adenylate ADP allows for additional proton flux through ATP synthase, which increased the respiratory rate. The addition of the substrates glutamate and succinate provided the substrates for complex II and resulted in increased respiratory rate (Fig. 3). Artificially induced leak respiration with the uncoupler CCCP resulted in negligible change in respiration. The inhibition of electron flux through complex I with rotenone leaves only electron flux through complex II to support oxidative phosphorylation. The introduction of ascorbate and TMPD provide reducing agents to subvert the TCA cycle and maximize ETS flux. The difference between this value and complex IV inhibited with azide provides complex IV's flux contribution (Fig. 3).

There was a trend for the routine respiratory rate to be higher in the control than in the T2D, but it did not reach statistical significance ($p = .094$; Fig. 3). Leak respiration was significantly higher for control compared to pre-treatment T2D ($p = .009$; Fig. 3). There was no difference between control and T2D in the permeabolized, complex I, OXPHOS, ETS, complex

II, or complex IV respiratory states. From pre to post-treatment, there were no significant changes in any of the respiratory states in the subjects given this isoleucine supplement for ten days.

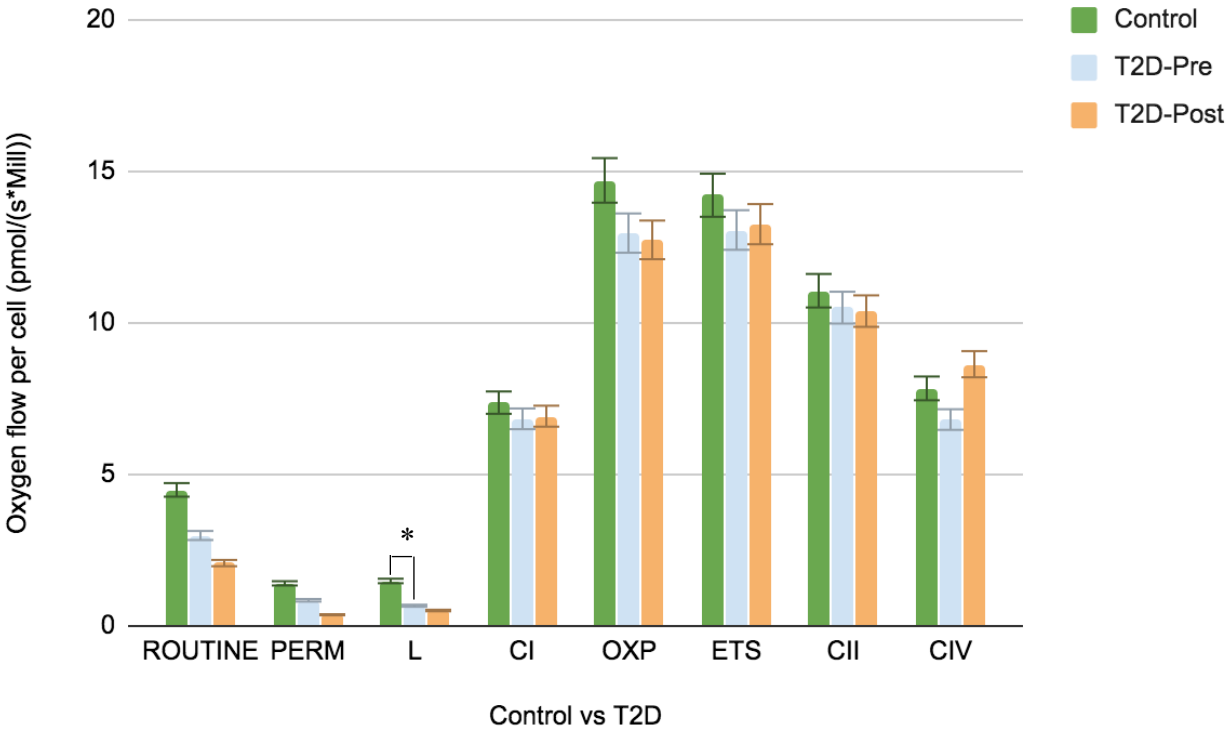

Figure 3: Respiratory Rates. Oxygen flow per cell in the Routine, permeabilized, leak and oxidative phosphorylation states. Relative oxygen flux measured using high resolution respirometry. Average rates were taken from controls and T2D subjects before and after three grams of isoleucine supplementation for 10 days.

Coupling control ratio (CCR) was significantly greater in control subjects than T2D ($p = .027$; Fig. 4). Neither substrate control ratio (SCR) nor respiratory control ratio (RCR) were significantly different between the control and T2D. None of these respiratory ratios changed significantly with isoleucine treatment for T2D subjects (Fig. 4). The only significant difference detected included greater leak respiration and coupling control ratio in the control compared to T2D (Figs. 3 and 4).

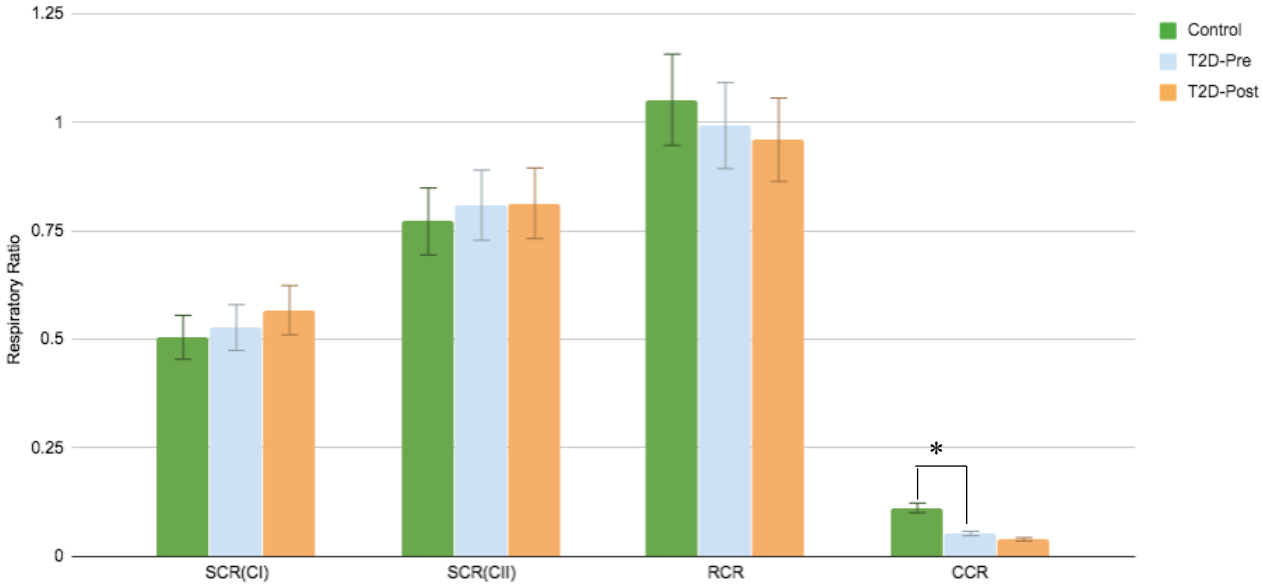

Figure 4: Respiratory Ratios. Controls and T2D subjects before and after treatment, respectively. These values were not measured in oxygen flow per cell, but are measured in a ratio instead. The respiratory ratios were calculated from the respiratory rate values. SCR_{CI} = CI / OXPHOS and SCR_{CII} = CII / OXPHOS. CCR = LEAK / OXPHOS. RCR = OXPHOS / ETS. Average rates were taken from controls and T2D subjects before and after three grams of isoleucine supplementation for 10 days.

CHAPTER IV

DISCUSSION

Respiratory States

After PBMCs were introduced and equilibrated to temperature and pressure in the high resolution respirometry chamber, they established native steady state routine respiratory rates assisted by intracellular substrates. After permeabolization with digitonin, substrates and adenylates used to support cellular respiration were washed out of the cell resulting in decreased respiration (Fig. 3). With induced leak through the reintroduction of the substrates pyruvate and malate, there was no detectable change in the respiratory rate from the permeabolized state. In the leak state, there are no adenylates for proton flux through ATP synthase, leaving leak as the only source of proton flux back to the matrix. Leak level is low, indicating that leak is not a significant portion of respiration in PBMCs. To induce maximum complex I respiration, the adenylate ADP was added to allow for proton flux through ATP synthase, resulting in increased respiratory rate. Glutamate and succinate were introduced to support electron flux through complex II, which induces maximum OXPHOS respiration with electron flux through complexes I and II, proton flux through ATP synthase, and uncoupled leak respiration. ETS respiration was induced to artificially increase the leak by creating an additional place for electrons to filter through, elevating the proton flux. ETS respiration did not increase past the level of OXPHOS, indicating that the ETC of PBMCs is efficient since it did not have the capacity for higher respiration past OXPHOS (Fig. 3).

Control vs. T2D and Treatment

The goal of this experiment was to determine if isoleucine was an effective treatment for the PBMC mitochondrial dysfunction in subjects with T2D. Based on previous research conducted with other inflammatory conditions, it was hypothesized that isoleucine may improve mitochondrial function in subjects with T2D. Leak respiration made up a minor portion of OXPHOS in the calculated CCR value, suggesting that leak is not a significant portion of respiration in PBMCs (Figs. 3 and 4). Although it was a minor portion of overall respiratory capacity, PBMC leak respiration and CCR were both significantly greater in healthy control than T2D subjects. These measures are related to each other in that CCR uses the leak respiratory rate to calculate its final value. Leak respiration is thought to reduce the production of reactive oxygen species and subsequent cellular damage (Zorov et al. 2014). Leak respiration in PBMCs from control subjects was higher compared to T2D, and thus control subjects may be more capable of regulating reactive oxygen species production (Fig. 3). While these differences showed up as significant, this trend may not necessarily hold with a larger sample population. However, our protocols proved to be effective with high repeatability in respiratory measures.

It was apparent that this specific treatment of isoleucine supplementation did not alter PBMC respiratory rates in subjects with T2D. In examining potential direction forward with this experiment, it is important to understand why this treatment could potentially alter PBMC respiration. A favorable result would improve oxygen flow in T2D subjects with administration of the isoleucine supplement. If a successful outcome were to take place, indicating a statistically significant difference in the respiratory rates between the pre and post-treatment in T2D subjects, this would mean that the oxidative capacity had increased. Oxidative capacity is known to increase with exercise and is correlated with a greater number of mitochondrial

proteins being expressed (Irving et al., 2015). Exercise capacity is a predictive indicator of metabolic health and coincides with low blood pressure and greater insulin sensitivity (Overmyer et al., 2015). With these effects, it can be determined that greater oxidative capacity is indicative of a more efficient functioning metabolism. Since chronic systemic inflammation is one of the primary characteristics of T2D that correlates with less efficient metabolic processes, it can be concluded that greater oxidative capacity parallels reduced inflammation. So, if this treatment had yielded increased oxidative capacity with the post-T2D group, it would indicate that the isoleucine supplement positively impacted the metabolism in these subjects (March et al., 2002). Succinyl-CoA in the TCA cycle is derived from isoleucine (March et al., 2002). In this anaplerotic role, isoleucine provides the necessary precursor biomolecules for succinyl-CoA to further the TCA cycle. This in turn provides electron flux to the ETC, with the ultimate goal of generating energy and yielding increased oxidative capacity.

Study Limitations

A variety of limitations may have affected this research including small sample size. The initial period of sample collection was used to develop experimental methodology and refine the experimental techniques and procedures to produce more accurate and consistent results. As such, the first five subjects were not included in the data analysis, leading to lower statistical power. Interruption in the supply of metabolic tracers used in other aspects of this study resulted in an unforeseen pause in new subject recruitment and limited the sample size of these preliminary results. An additional source of potential error is related to potential RBC contamination in the isolated mononuclear cells because this would artificially inflate the cell count. A cell count that is higher than reality would correspond to a higher expected oxidative capacity. This would parallel a seemingly low respiratory rate because the true cell count is

lower, meaning there are less cells with the capability to facilitate mitochondrial respiration. Another limitation was that the subjects were home when they were taking the supplement. Because of this, there was no true way of knowing whether or not they took the supplement as directed because their compliance and follow through was self-reported.

Next Steps

The presented results are preliminary from ongoing research and motivate multiple next steps to expand and further the effective scope of this research. The PBMCs measured as a marker for T2D mitochondrial function in this study are only one marker of this disease. Although isoleucine supplementation did not alter respiratory capacity in PBMCs, these results do not indicate that mitochondrial function was not affected in other tissues. While PBMCs were used to analyze oxidative capacity because of their minimal invasiveness, it may be beneficial to conduct a muscle biopsy to assess skeletal muscle mitochondrial function. This would allow for determination of whether isoleucine's effects are insignificant only in the PBMC measure analyzed in this study, or if these affects are true of overall physiological function. To further verify these results, it would be interesting to assess the measures of the subjects' blood glucose levels, blood pressure, or other indications of T2D. These values were taken, but as a part of a different study. This would allow for the assessment of the treatment's effect on the cellular oxidative levels, from the PBMC study we conducted, and also the disease symptoms that current T2D address.

In addition, isoleucine is one branched chain amino acid, a part of the overarching group that has been shown to positively affect mitochondrial function in other chronic inflammatory conditions. It might be productive to conduct a similar study with other BCAAs including valine and leucine. A proposed future step is to look at the effects of valine because, compared to

leucine, valine's structure varies more from that of isoleucine. If valine were to induce a significant change in the respiratory capacity, this would provide data about effective nutritional supplement interventions for T2D. Another proposed future research study would involve conducting the study for a longer duration. Although 10 days of isoleucine supplementation did not alter PBMC respiration in subjects with T2D, longer duration supplementation may be required to invoke change.

Conclusion

We were able to develop protocols to collect consistent and repeatable HRR measures in isolated PBMC from whole blood. PBMC leak respiration and CCR were both significantly greater in control than T2D subjects. However, isoleucine treatment did not alter the respiratory rates to be more similar to control measures.

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