DEVELOPMENT OF A METHOD TO INCREASE THE FLUX OF METABOLISM OF HEPATOCYTES *IN VITRO*

by

ELIZABETH WOOD

(Under the Direction of Chad Paton)

ABSTRACT

In vitro models of metabolism are powerful tools for discovery, however they are limited by their relatively low metabolic activity and energy expenditure. Metabolic flexibility is a key component of a healthy liver. Dysregulation of hepatic metabolism is linked with lipid accumulation, insulin resistance, and diabetes, due in part to diminished energy expenditure and a lack of AMP-activated protein kinase (AMPK) activity. The primary objective of this research is to develop a model to increase the flux of metabolism in FL83B cells *in vitro* using beta-guanidinopropionic acid (β-GPA), 1-β-D-ribofuranoside 5-amino-imidazole-4-carboxamide ribose (AICAR), or carbachol. Acute AICAR treatment significantly increased the ADP:ATP ratio, but this impact did not result in acute changes in metabolic flux or chronic changes in gene expression. Acute β-GPA and carbachol treatments did not increase metabolic demand, but chronically, both β-GPA and carbachol increased PGC-1α and perilipin-2 expression.

INDEX WORDS: Metabolism, AICAR, β-GPA, Carbachol, Hepatocytes, AMPK, Metabolic Flux, FL83B

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ELIZABETH WOOD

BS, University of Wisconsin-Stout, 2018

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2020

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ELIZABETH WOOD

Major Professor:	Chad Paton
Committee:	Jarrod Call
	Jamie Cooper

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
August 2020

ACKNOWLEDGEMENTS

Funding for this project was provided by the University of Georgia Obesity Initiative. I would like to thank my lab mates, Yura Son, Jo Shieh, and Mai Spaulding, for all of their help, support, and encouragement. I would also like to thank the Call Lab (University of Georgia Kinesiology) and Jennifer McFaline-Figueroa for their help.

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CHAPTER 1

INTRODUCTION

Metabolic flexibility, the ability to respond to metabolic demand, is a key component of a healthy liver and is associated with efficient fuel utilization and glycemic control [1, 2]. Dysregulation of hepatic metabolism and metabolic flexibility is linked with lipid accumulation, insulin resistance, and diabetes, but increasing metabolic demand and/or activating the AMP-activated protein kinase (AMPK) pathway can help prevent or improve these conditions [3, 4]. Methods that allow us to study these processes by increasing energy expenditure *in vitro*, such as the use of exercise mimetics or pharmacological AMPK activators, are important tools since the metabolic demand of cell culture does not compare to that of living organisms. Developing such methods provide the opportunity to show proof of concept and understand the physiological mechanisms at a basic level, which can help improve liver function and health.

Background

The liver is an essential and metabolically versatile digestive organ and the first organ to which most absorbed nutrients are transported [5]. It is involved in metabolizing, storing, and supplying nutrients for the whole body [1]. Given the critical nature of the liver, proper metabolic function is necessary for overall health, as dysregulation of its metabolic functions is associated with disease states like nonalcoholic fatty liver disease (NAFLD) and insulin resistance [1].

Maintaining energy homeostasis and metabolic function helps prevent hepatic fat accumulation, which is also linked with insulin resistance and inflammation [3]. This is

significant because increased fat content of the liver can increase risk for cardiovascular disease, type 2 diabetes, and liver failure [3, 6]. Treating and preventing excess hepatic fat buildup involves reducing fat accumulation and increasing energy expenditure and the flux of metabolism, which are pathways that involve AMPK [3, 7]. Activation of AMPK and its downstream targets may be beneficial for enhancing fuel utilization and maintaining energy and metabolic homeostasis.

AMPK Pathway

The AMPK pathway acts as a metabolic and energy sensor within cells [5, 8, 9]. When a cell is depleted of adenosine triphosphate (ATP) or ATP consumption increases, ATP concentrations drop and AMP concentrations rise, thus activating AMPK [10, 11]. AMPK works to regulate cellular energy levels by stimulating catabolic processes that produce ATP while decreasing anabolic processes that consume ATP, which allows cells to adapt to increased energy needs [5, 11].

Several factors and conditions have been shown to activate AMPK, including adiponectin, energy deprivation, exercise, hypoxia, muscarinic receptor activation, and the pharmacological activator 1-β-D-ribofuranoside 5-amino-imidazole-4-carboxamide ribose (AICAR) [11-17]. On the other hand, conditions such as obesity have been show to impair AMPK function in skeletal muscle, and increased adiposity and high-fat diets can decrease activated AMPK levels in the liver [18, 19].

Pharmacological treatments for increasing the flux of metabolism in vitro

AICAR

AICAR is an AMP analog and AMPK activator. As an AMPK activator, AICAR treatment can up-regulate fatty acid oxidation [20, 21]. *In vitro* AICAR treatment significantly

increases AMPK activation within 15 minutes of treatment and inhibits enzymes involved in fatty acid and cholesterol synthesis, which are pathways down regulated with AMPK activation [15]. Furthermore, in isolated rat hepatocytes, AICAR treatment has been shown to decrease glucokinase activity and levels of glucose-6-phosphate, fructose-6-phosphate, and fructose 2,6-bisphosphate, suggesting decreased glycolysis [22]. Decreased glycolysis activity was also observed by Guigas et al (2006), where AICAR activated AMPK and inhibited hepatic glucose phosphorylation [16]. However, this down-regulation of glycolysis is suspected to be through an AMPK-independent mechanism since AMPK phosphorylation did not affect the activity of phosphorylated 6-phosphofructokianse-2/fructose-2,6,-bisphosphatase [16]. Although glucose utilization is down regulated, hepatic fatty acid oxidation is increase with AICAR administration, further indicating a shift from glucose to lipid oxidation with AMPK activation [23].

β-GPA

Beta-guanidinopropionic acid (β-GPA) is a creatine analog that reduces creatine uptake within cells, which prevents the cells from forming phosphocreatine for energy [24]. Creatine kinase, which catalyzes the reversible reaction of creatine to/from phosphocreatine coupled with the phosphorylation/dephosphorylation of ATP to/from adenosine diphosphate (ADP), helps regulate cellular energy levels by allowing for rapid cytosolic or mitochondrial ATP production, depending on the M or B isoform [25, 26].

However, creatine kinase has low activity in the liver, and literature describing the effect of β-GPA treatment in hepatocytes is scarce [27]. Nonetheless, *in vivo* β-GPA treatment in obese and diabetic mice and rhesus monkeys decreases body weight of mice and elicits an anti-hyperglycemic effect with reduced blood glucose levels compared to control animals [28]. Baumgarner and colleagues (2015) also showed reduced body weight in β-GPA-treated mice in

addition to increased active AMPK in skeletal muscle, and Ohira and colleagues (1994) also found improved glucose tolerance in β-GPA-supplemented rats [29, 30]. Moreover, *in vivo* β-GPA supplementation in rats has been shown to increase liver and skeletal muscle glycogen content, indicating an effect on carbohydrate metabolism and utilization [30]. In a mouse model of Huntington's Disease, Chaturvedi et al (2010) examined the effects β-GPA in the brain, liver, and brown adipose tissue [31]. β-GPA treatment increased PGC-1α, mitochondrial transcription factor A (Tfam), and hepatocyte nuclear factor alpha (HNFα) expression in the liver of wild type mice, suggesting a role of β-GPA in increasing hepatocyte mitochondrial content [31].

Carbachol

Carbachol is an acetylcholine analog and muscarinic receptor agonist [14]. Muscarinic receptors are acetylcholine receptors that are expressed in the liver, specifically the subtypes M1 and M3 [14, 32]. In the liver, these receptors mediate metabolic responses to acetylcholine, especially changes in glucose metabolism [33]. While investigating the role of M3 muscarinic receptors in acetylcholine-induced metabolic changes, Vatamaniuk and colleagues (2003) found that acetylcholine perfusion in rat liver increased oxygen consumption and glucose production [33]. However, the addition of atropine, a muscarinic receptor inhibitor, repressed these acetylcholine-induced effects, suggesting the key role of muscarinic receptors in mediating metabolic changes in the liver [33]. Moreover, Jadeja et al (2019) found that muscarinic activation with carbachol in an *in vitro* steatosis model decreases lipid accumulation and activates AMPK [14]. In an in *vivo* carbachol infusion model, carbachol treatment has been shown to promote hepatocyte growth [34].

Research Question

Which pharmacological treatment (β-GPA, AICAR, or carbachol) enhances the flux of metabolism to the greatest extent in murine liver cell culture?

Hypothesis

We hypothesized that energy expenditure and oxidative metabolism can be increased *in vitro* through the use of β-GPA, AICAR, or carbachol.

Primary Objective and Specific Aims

The primary objective of this project was to develop a model to increase oxidative metabolism in murine liver cells. Our first specific aim was to determine which treatment method (β-GPA, AICAR, or carbachol) results in a change in energy status in cell culture. Our second specific aim was to determine which treatment method results in the greatest increase in the expression of genes associated with oxidative metabolism.

Rationale and Significance

Cells in culture do not have a high energy expenditure at baseline, and to our knowledge, no other studies have been conducted to examine methods for increasing energy expenditure and the flux of metabolism in liver cells *in vitro*. Therefore, the purpose of this study was to develop a method to most effectively model energy expenditure *in vitro* for proof of concept, to generate new hypotheses, and to drive future research questions. This research is significant because it is beneficial for targeting and improving hepatic fuel utilization and metabolism, which can improve health outcomes.

CHAPTER 2

REVIEW OF THE LITERATURE

Introduction

The liver is an essential and metabolically versatile accessory digestive organ [5]. The liver is the first organ to which most absorbed nutrients are transported and is involved in metabolizing, storing, and supplying nutrients in addition to maintaining whole-body glucose homeostasis through glycogen storage and gluconeogenesis [1]. Given the critical nature of the liver, proper function is necessary for overall health, as dysregulation of its metabolic functions is associated with disease states like nonalcoholic fatty liver disease (NAFLD) and insulin resistance [1].

Maintaining energy homeostasis and metabolic function helps prevent hepatic fat accumulation, which is also linked with reduced insulin sensitivity and inflammation [3]. This is significant because increased fat content of the liver can increase risk for cardiovascular disease, type 2 diabetes, and liver failure [3, 6]. Treating and preventing excess hepatic fat buildup involves reducing fat accumulation as well as increasing energy expenditure and enhancing the flux of metabolism, which are pathways that involve AMP-activated protein kinase (AMPK) [3, 7]. Activation of AMPK and its downstream targets may be beneficial for enhancing fuel utilization and maintaining energy and metabolic homeostasis.

AMPK Pathway

The AMP-activated protein kinase (AMPK) pathway acts as a metabolic and energy sensor within cells [5, 8, 9]. When a cell is depleted of ATP or ATP consumption increases, ATP

concentrations drop and AMP concentrations rise, thus activating AMPK [10, 11]. AMPK works to regulate cellular energy levels by stimulating catabolic processes that produce ATP while decreasing anabolic processes that consume ATP, which allows cells to adapt to increased energy needs [5, 11].

Several factors and conditions have been shown to activate AMPK, including adiponectin, energy deprivation, exercise, hypoxia, muscarinic receptor activation, and the pharmacological activator 1-β-D-ribofuranoside 5-amino-imidazole-4-carboxamide ribose (AICAR) [11-17]. On the other hand, conditions such as obesity have been show to impair AMPK function in skeletal muscle, and increased adiposity and high-fat diets can decrease activated AMPK levels in the liver [18, 19].

Roles of AMPK in the cell

AMPK has several important roles in cells. It regulates energy homeostasis, carbohydrate and glucose metabolism, responses to exercise, and transcriptional activities of metabolic genes [5, 8, 13, 16, 35]. In terms of carbohydrate metabolism, AMPK down-regulates gluconeogenic proteins, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase [36, 37]. Down-regulation of gluconeogenic proteins prevents the biosynthesis of glucose, an ATP-consuming process, in effort to increase ATP production for energy homeostasis. This inhibition of endogenous glucose synthesis has effects at the organismal level, as hepatic overexpression of AMPK has been shown to reduce blood glucose concentrations in obese, diabetic, and normal mice [36]. This is supported by the finding that dominant-negative hepatic AMPK prevents gluconeogenesis inhibition [12]. Glycemic effects of AMPK activation are not only seen in the liver, but also skeletal muscle. AMPK activation in skeletal muscle increases glucose uptake, but

this increase in uptake and transport is reduced in transgenic mice with inactive skeletal muscle AMPK [38].

Lipid metabolism is also a target of AMPK. Similar to the inhibition of glucose synthesis, fatty acid synthesis is also inhibited with AMPK activation [14, 15, 36]. Acetyl CoA carboxylase (ACC) regulates fatty acid synthesis and oxidation by catalyzing the reaction that produces malonyl CoA, which inhibits carnitine palmitoyl transferase-1 (CPT-1). ACC is inactive in the phosphorylated state and active in the dephosphorylated state. AICAR-induced activation of AMPK increases phosphorylation of ACC in isolated hepatocytes of both starved and fed rats, and the same effect was seen in AMPK-activated murine hepatocytes and hepatoma cells [14, 16]. Hepatic overexpression of AMPK has also been shown to inactivate ACC through increased phosphorylation and reduce ACC expression in the liver [36, 37]. Decreased ACC activity has also been shown in AICAR-induced AMPK activation in rat skeletal muscle [20].

Along with reduced glucose and fatty acid synthesis, increased fatty acid β-oxidation is a downstream effect of AMPK activation [39]. *In vivo* hepatic AMPK activation has been shown to increase fatty acid uptake and decrease circulating lipid concentrations [39]. Enhanced β-oxidation and fatty acid utilization from AMPK activation also prevents lipid accumulation in the liver and can decrease body fat mass [9, 14, 39, 40]. In contrast to studies showing decreased hepatic lipid content, Foretz et al (2005) found increased cholesterol and triglycerides in the livers of mice acutely overexpressing hepatic AMPKα2 through adenovirus infection [36]. The same investigation also showed increased lipid utilization, decreased lipogenesis, and decreased body fat in the AMPK-infected mice, indicating that the increased hepatic triglyceride content may serve as an energy source [36]. Nonetheless, activation of AMPK shifts metabolic pathways toward lipid oxidation [36, 39].

AMPK is also recognized as a key regulator of mitochondrial biogenesis [41]. Increased mitochondria content allows for greater fatty acid oxidation, and studies in skeletal muscle show that AMPK is necessary for mitochondrial biogenesis [42]. Zong et al (2002) compared wild-type mice to mice with a dominant-negative mutant of AMPK in skeletal muscle to identify its role in the process of mitochondrial biogenesis. In response to chronic energy deprivation, AMPK was activated and mitochondrial biogenesis was increased in wild-type mice, but not in AMPK-mutant mice [42]. Furthermore, regarding hepatic mitochondrial function, AICAR-induced AMPK activation normalized mitochondrial function and fusion in a drug-induced hepatocyte injury model that caused mitochondrial dysfunction [43].

Given the significant role of AMPK in sensing and regulating energy status in cells, Zong and colleagues (2002) argue that activation of AMPK is the initial step in the regulation of cellular metabolic responses [42]. AMPK enhances ATP production and induces downstream transcriptional regulation, such as that of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) [8].

AMPK and PGC-1α

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcriptional co-activator and a key component in the transcriptional regulation of metabolism and mitochondrial biogenesis. PGC-1α is expressed in highly metabolic tissues, such as the liver, skeletal muscle, and heart [8]. In the liver, PGC-1α is up-regulated during states of fasting and low insulin levels, similar to conditions that activate AMPK [11, 44-46]. In hepatocytes with adenovirus-infected active AMPK, PGC-1α expression is increased, and hepatocytes treated with metformin, a known AMPK activator, also show increased PGC-1α expression with AMPK activation [47]. Similar effects are seen in skeletal muscle. *In vivo* skeletal muscle AMPK

activation increases PGC-1α expression, and pharmacological AMPK activation fails to increase PGC-1α in skeletal muscle with inactive AMPK [42, 48]. However, in hepatocytes, AICAR-mediated AMPK activation has been shown to down-regulate PGC-1α [47]. This effect of AICAR could be independent of AMPK activation, given that other known AMPK activators, like fasting and Metformin, increase PGC-1α expression [47].

PPARδ

Peroxisome proliferator activated receptor delta (PPARδ) is a ligand-regulated nuclear receptor (i.e. DNA binding transcription factor) and a regulator of hepatic carbohydrate and lipid metabolism [49, 50]. In investigation into the role of PPARδ in liver metabolism, Liu et al (2011) used adenovirus mediated PPARδ infection in the liver of mice [49]. PPARδ-overexpressed mice showed enhanced insulin sensitivity, glucose utilization, and AMPK activation with elevated levels of phosphorylated AMPK [49]. These mice also had increased GLUT2 and glucokinase expression, suggesting enhanced glucose uptake and a beneficial role of PPARδ in hepatic metabolic activity [49]. Similar results were found in diabetic mice treated with GW501516, a known PPARδ agonist. PPARδ agonism improved glucose tolerance and insulin sensitivity, but increased hepatic triglyceride concentrations [50]. Liu et al (2011) also reported changes in hepatic lipids, specifically increased monounsaturated fatty acids (18:1) and decreased saturated fatty acids (16:0), in livers of mice overexpressing PPARδ [49].

In skeletal muscle and adipose tissue, PPARδ enhances fatty acid β-oxidation [50, 51]. Kleiner et al (2009) found that pharmacological activation via GW501516 treatment activated mitochondrial fatty acid oxidation genes, including CPT-1b, pyruvate dehydrogenase kinase 4 (PDK4), and uncoupling protein 3 (UCP3) [52]. Taken together, PPARδ plays a key role in

hepatic glucose utilization, impacts lipid metabolism, and increases expression parallel with AMPK.

PPARα

Peroxisome proliferator activated receptor alpha (PPARα) is another ligand-regulated nuclear receptor that is expressed and regulates lipid metabolism in the liver, particularly in the fasted state [14, 53]. Several studies show impaired lipid metabolism in PPARα-null mice [54-58]. PPARα-null mice exhibit decreased fatty acid uptake and oxidation, hepatic fat accumulation, impaired gluconeogenesis, and hypoglycemia [54, 55, 57]. This shows that PPARα is critical to mediate the adaptations to fasting, specifically regarding lipid metabolism, and Vega and colleagues (2000) demonstrate that it does so with PGC-1 [59]. Co-retrovirus-induced expression of both PGC-1 and PPARα increase mitochondrial oxidation proteins to a greater extent than each expression individually [59]. AICAR-mediated AMPK activation has been shown to increase PPARα expression and the expression of PPARα target genes, including PGC-1 [57]. This suggests interaction between AMPK, PPARα, and PGC-1 that mediates metabolic pathways during fasting or low energy availability.

Treatments for Increasing the Flux of Metabolism

AICAR

1-β-D-ribofuranoside 5-amino-imidazole-4-carboxamide ribose (AICAR) is an AMP analog and AMPK activator. As an AMPK activator, AICAR treatment can up-regulate fatty acid oxidation [20, 21]. *In vitro* AICAR treatment significantly increases AMPK activation within 15 minutes of treatment and inhibits enzymes involved in fatty acid and cholesterol synthesis, which are pathways down regulated with AMPK activation [15]. Furthermore, in isolated rat hepatocytes, AICAR treatment has been shown to decrease glucokinase activity and

levels of glucose-6-phosphate, fructose-6-phosphate, and fructose 2,6-bisphosphate, suggesting decreased glycolysis [22]. Decreased glycolysis activity was also observed by Guigas et al (2006), where AICAR activated AMPK and inhibited hepatic glucose phosphorylation [16]. However, this down-regulation of glycolysis is suspected to be through an AMPK-independent mechanism since AMPK phosphorylation did not affect the activity of phosphorylated 6-phosphofructokianse-2/fructose-2,6,-bisphosphatase [16]. Although glucose utilization is down regulated, hepatic fatty acid oxidation is increased with AICAR administration, further indicating a shift from glucose to lipid oxidation with AMPK activation [23].

AICAR also has metabolic effects in skeletal muscle. AICAR-treated mice have been shown to have greater endurance compared to controls, and these effects are induced through activation of the AMPK and PPARδ pathways [21]**.** Jager et al (2007) found that AICAR treatment in primary myotubes increased both total and uncoupled respiration, and similar results were found when Bueno Junior and colleagues (2012) showed that AICAR treatment combined with exercise in mice significantly improved oxidative capacity compared to controls while also increasing the number of type I (oxidative) muscle fibers and cross-sectional area of skeletal muscle [60, 61]. Furthermore, AICAR treatment increases glucose uptake in skeletal muscle while increasing citric acid cycle and β-oxidation pathways in a glucose-sparing mechanism [20, 62].

β-GPA

Beta-guanidinopropionic acid (β-GPA) is a creatine analog that reduces creatine uptake within cells, which prevents the cells from forming phosphocreatine for energy [24]. Creatine kinase, which catalyzes the reversible reaction of creatine to/from phosphocreatine coupled with the phosphorylation/dephosphorylation of ATP to/from ADP (Figure 1), helps regulate cellular

energy levels by allowing for rapid cytosolic or mitochondrial ATP production, depending on the M or B isoform [25, 26].

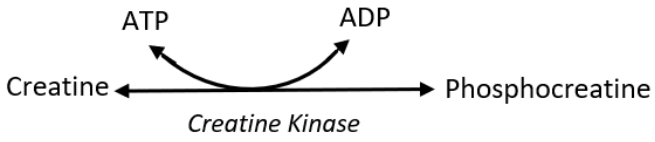

Figure 1. Creatine kinase reaction.

β-GPA and phosphorylated β-GPA are poor substrates for creatine kinase and become competitive inhibitors of this enzyme [10, 63]. β-GPA is unable to be utilized by mitochondria, does not stimulate mitochondrial respiration, is poorly phosphorylated by mitochondrial creatine kinase, and cannot be a substitute for creatine [24]. In skeletal muscle, the inefficiency of β-GPA as a substrate for creatine kinase has been shown both *in vitro* and *in vivo*, and as a result, causes a significant reduction in ATP, phosphocreatine, and creatine and activates AMPK [10, 48, 63-65].

However, creatine kinase has low activity in the liver, and literature with β-GPA treatment to hepatocytes is scarce [27]. Nonetheless, *in vivo* β-GPA treatment in obese and diabetic mice and rhesus monkeys decreases body weight of mice and elicits an anti-hyperglycemic effect with reduced blood glucose levels compared to control animals [28]. Baumgarner and colleagues (2015) also showed reduced body weight in β-GPA-treated mice in addition to increased active AMPK in skeletal muscle, and Ohira and colleagues (1994) also found improved glucose tolerance in β-GPA-supplemented rats [29, 30]. Moreover, *in vivo* β-

GPA supplementation in rats has been shown to increase liver and skeletal muscle glycogen content, indicating an effect on carbohydrate metabolism and utilization [30]. In a mouse model of Huntington's Disease, Chaturvedi et al (2010) examined the effects β-GPA in the brain, liver, and brown adipose tissue [31]. β-GPA treatment increased PGC-1α, mitochondrial transcription factor A (Tfam), and hepatocyte nuclear factor alpha (HNFα) expression in the liver of wild type mice, suggesting a role of β-GPA in increasing hepatocyte mitochondrial content [31].

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Regarding carbachol treatment, Jadeja et al (2019) found that muscarinic activation with carbachol in an *in vitro* steatosis model decreases lipid accumulation and activates AMPK [14]. As discussed previously, AMPK activation induces numerous adaptive downstream effects, including mitochondrial biogenesis and enhanced lipid oxidation. In an in *vivo* carbachol infusion model, carbachol treatment was been shown to promote hepatocyte growth [34].

Migliorini et al (1989) used intraventricular carbachol injection in rats to study its metabolic effects and found inhibited pyruvate kinase activity, indicating reduced glycolysis activity, an effect also seen with AICAR treatment [22, 67]. Moreover, the same study with carbachol injection showed increased plasma glucose and lactate concentrations, which was induced by increased hepatic gluconeogenesis and glucose release from the liver [67]. Increased gluconeogenesis is not seen with hepatic AMPK activation; however, Migliorini et al did not examine whether this effect was associated with AMPK activity [67].

Carbachol also affects skeletal muscle. As an acetylcholine receptor agonist, carbachol induces both oxidative and glycolytic skeletal muscle contraction [68, 69]. Because of its ability to induce muscle contraction, carbachol has been shown to activate AMPK, increase intracellular calcium levels *in vitro*, increase glucose uptake via GLUT4, and increase mitochondrial biogenesis [70-72]**.**

TARGET GENES

Perilipin 2

Perilipin 2 is a lipid droplet-coating protein expressed in various tissues, including liver and skeletal muscle [73, 74]. Lipid droplets are lipid-storing molecules that regulate lipid utilization within cells, and perilipin 2 levels generally indicate lipid droplet levels [73, 75]. Perilipin 2 expression has been shown to be increased by reactive oxygen species, exercise training, and exposure to high concentrations of fatty acids [74, 76]. Several studies show beneficial effects of perilipin 2 deletion. Tsai and colleagues (2017) showed that perilipin 2-null mice have significantly reduced hepatic triglyceride content, and Libby and colleagues (2016) demonstrated that perilipin 2-null mice do not become obese or insulin resistant with reduced hepatic triglycerides on a 30-week Western diet [73, 77]. Similar results were found with liver-

specific perilipin 2 knockout mice with experimentally induced fatty liver. These mice without hepatic perilipin 2 had decreased fat buildup and inflammation in addition to decreased serum lipoprotein levels [78]. Furthermore, hepatic perilipin 2-deficient mice have increased expression of β-oxidation genes, indicating increased fatty acid oxidation [78]. On the other hand, overexpression of perilipin 2 prevents lipid droplet breakdown [73]. Perilipin 2-null mice have also been shown to have increased levels of activated AMPK when fed a high-fat diet with decreased levels of lipogenic proteins [77].

Similar findings have been shown in skeletal muscle. Using skeletal muscle cultures from perilipin-2-WT and -KO mice, Feng et al (2017) found that perilpin-2-KO myotubes had lower storage amounts of lipid droplets compared to perilipin-2-positive myotubes [79]. Moreover, perilipin-2-null cells show increased lipolysis and fatty acid oxidation and preferentially use fatty acids for energy rather than glucose [79]. Taken together, perilipin 2 plays a critical role in regulating lipid storage and metabolism in both liver and muscle, and deletion of perilipin 2 induces both hepatic and organismal level changes in adiposity and lipid metabolism.

PGC-1α

As discussed previously, PGC-1α is a key regulator of metabolism and mitochondrial biogenesis and can be up-regulated in the liver by fasting and low insulin levels [44-46]**.** *Hepatic* PGC-1α overexpression has been shown to increase mitochondrial DNA and fatty acid β-oxidation and decrease hepatic triglyceride buildup [80]. Increased mitochondrial content is accompanied by enhanced mitochondrial function with PGC-1α expression, as PGC-1α overexpression also increases mitochondrial electron transport chain proteins and enzymes [80]. Moreover, PGC-1α has been shown to regulate and increase gluconeogenic proteins, which could indicate a response to low blood glucose from fasting or starvation [44, 81]. PGC-1α,

similar to AMPK activation, stimulates hepatic lipid oxidation, which is also seen in skeletal muscle [52, 80, 81]. PGC-1α may also play a role in inflammation. Mice on a 10-week high-fat diet exhibit increased pro-inflammatory cytokine levels (IL-6, TNF-α) and decreased PGC-1α expression in the liver, an effect also seen in PGC-1α knockdown HepG2 cells [82]. Similar roles in inflammation are also seen in astrocytes, where increased PGC-1α reduces inflammatory markers, and skeletal muscle, where PGC-1α knock-out mice show increased inflammatory markers following exercise [83, 84].

Hepatic triglyceride accumulation could negatively affect PGC-1α activity. Aharoni et al (2011) showed that fatty liver induced in mice resulted in an increase in PGC-1α mRNA expression in the fasted state, but mitochondrial biogenesis was impaired with lower mitochondrial content [85]. Yoon et al (2001) also found increased hepatic PGC-1α mRNA in obese mice compared to non-obese controls [44]. Collectively, PGC-1α plays a role in both lipid and glucose metabolism, is a key factor of mitochondrial biogenesis, and mediates responses to changes in energy status or needs.

Conclusion

The liver is an essential metabolic organ with functions that affect the whole organism. Although dysregulation of liver metabolism can negatively impact its function, AMPK activation and treatments with AICAR, β-GPA, and carbachol help improve mitochondrial biogenesis, fatty acid β-oxidation, and glucose tolerance while decreasing lipid accumulation in the liver. With each treatment (AICAR, β-GPA, and carbachol) showing effects *in vitro* and *in vivo* in various tissues, developing a model that best demonstrates energy expenditure in hepatocytes allows us to study the metabolic processes at a basic level to enhance liver metabolic flexibility, function, and health.

CHAPTER 3

METHODS

Study Design

This project was a basic research study with a method-developing and hypothesis-generating design. Three different treatments, 1-β-D-ribofuranoside 5-amino-imidazole-4-carboxamide ribose (AICAR), β-guanidinopropionic acid (β-GPA), and carbachol, were used to investigate the most effective method to increase the flux of metabolism in hepatocytes *in vitro*. This study used cell culture and both acute and chronic treatments, and the effects of these treatments were measured through changes in gene expression, adenosine diphosphate/adenosine triphosphate (ADP/ATP) ratios, oxygen consumption, nicotinamide adenine dinucleotide (NAD^+/NADH) quantities, and changes in phospho-AMPK activity. An *in vitro* study design was selected because of the exploratory nature of this project, with the goal being to improve our knowledge and understanding of this topic at a basic level.

Cell Culture

Murine FL83B liver-like cells were obtained from ATCC (Manassas, VA, CRL2390) and used for this study. FL83B cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) (Corning Life Sciences, Tewksbury, MA) with 10% fetal bovine serum (FBS) (Corning Life Sciences, Tewksbury, MA) and 1% penicillin/streptomycin (P/S) (Genesee Scientific, San Diego, CA) and incubated at 37 degrees Celsius with 5% CO_2 until they were 80% confluent. Treatments were applied when the cells reach appropriate confluency.

Treatments

Cells were treated with 500µM AICAR (Santa Cruz Biotechnology, Dallas, TX; sc-200659), 20µM β-GPA (Sigma-Aldrich, St. Louis, MO; G6878), and 10µM carbachol (Tocris Bioscience, Minneapolis, MN; catalog #2810) for a time range of 0 to 16 hours. Ultra-pure H_2O was the vehicle for β-GPA, and DMSO was the vehicle for AICAR and carbachol in the same concentrations as β-GPA, AICAR, and carbachol. Ultra-pure H_2O and DMSO were also the control treatments.

Table 1. Treatment concentrations and times.

Treatment	**Concentration**	**Time**	
AICAR	500µM	Acute Treatments	Chronic Treatments
β-GPA	20µM	15 minutes	16 hours
Carbachol	10µM		

Outcome Measures

Gene Expression

The expression of genes involved in mitochondrial biogenesis and fatty acid oxidation were measured using quantitative real-time polymerase chain reaction (RT-PCR) following 16-hour treatments. mRNA was collected from cells with Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and a cDNA reverse transcription kit (Applied Biosystems, Grand Island,

NY, USA) was used for cDNA synthesis. StepOnePlus RT-PCR system with StepOne Software and SYBR Green Reagent was used to carry out this quantification. All primers were designed to span exon-exon boundaries and were obtained from Integrated DNA Technologies, Coralville, IA, USA. The RT-PCR primer sequences are listed in table 2.

Table 2. Primer sequences for Cyclophilin A (housekeeping), PGC-1α, and perilipin-2.

Primer	Sequence
Cyclophilin A	Forward: 5'-CGAGCTCTGAGCACTGGAGAGAAA-3'
	Reverse: 5'-ATGGACAAGATGCCAGGACCTGTA-3'
PGC-1α	Forward: 5'-AGCACTCAGAACCATGCAGCAAAC-3'
	Reverse: 5'-TTTGGTGTGAGGAGGGTCATCGTT-3'
Perilipin-2	Forward: 5'-CAGCAGGGTTAAAGAGGCCAAACA-3'
	Reverse: 5'-TTCCACTCCACCCACGAGACATAG-3'

Changes in phospho-AMPK Levels

Western Blot was used as described by Sampath et al (2009) to determine changes in enzyme activity of phospho-AMPK [86]. Cells were washed with pre-warmed phosphate-buffered saline (PBS) and incubated with pre-warmed serum-free DMEM with 1% P/S for 2 hours. Cells were then treated with pre-warmed serum-free DMEM containing vehicle, 20μM β-GPA, 500μM AICAR, or 10μM carbachol for 15 minutes. Cells were collected in ice-cold

radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail. Total protein concentration was measured using the Bradford assay and samples were normalized by protein concentration. The primary antibody P-AMPK (T172) was obtained from Cell Signaling Technology (Danvers, MA, USA), and beta-actin (I-19) was used as a reference protein and obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibodies (Cell Signaling, Beverly MA) were diluted in 5% BSA in 1X tris-buffered saline with TWEEN (1X TBST) and used to detect the proteins of interest. Following this, a horseradish peroxidase-conjugated anti-rabbit IgG-secondary antibody (Cell Signaling Technology, Danvers, MA, USA) was added. The membrane was developed with WesternSure premium chemiluminescent substrate (LI-COR bioscience, NE). ImageJ (NIH) was used to quantify band intensity.

Oxygen Consumption

Oxygen consumption was measured using high-resolution respirometry with Oroboros O2k oxygraphs (Oroboros Instruments, Innsbruck, Austria). Cells were grown in 6-well tissue-culture plates and re-suspended by trypsinization after confluency. Cells were then counted using a hemocytometer (Hausser Scientific, Horsham, PA) and diluted to 5×10^5 cells/mL. Cells were then added to the oxygraph chamber with constant stirring using a magnetic stir bar. 2 µL of 8.1 mM digitonin was added to permeabilize the cells. Vehicle treatment, 20µM β-GPA, 500µM AICAR, or 10µM carbachol was added for 15 minutes before the addition of substrates. Glutamate (10mM), malate (5mM), succinate (10mM), and ADP (5mM) were added to stimulate and measure state III respiration. Carbonyl cyanide-4-trifluoromethoxyphenylhydrazone (FCCP) was added to stimulated and measure uncoupled respiration. Oxygen consumption measurements were normalized to cell number.

ADP/ATP Ratios

The ADP/ATP ratio was determined using the ADP/ATP Ratio Assay Kit (Sigma-Aldrich, MO, USA; MAK135) per manufacturer's directions. Briefly, FL83B cells were grown in a 96-well tissue culture plate. Cells were washed with pre-warmed phosphate-buffered saline (PBS) and incubated with pre-warmed serum-free DMEM with 1% P/S for 2 hours. Cells were then treated with pre-warmed treatment media containing vehicle, 20μM β-GPA, 500μM AICAR, or 10μM carbachol for 15 minutes. The assay buffer, D-Luciferin, and the ATP enzyme were added to lyse the cells and ATP reacted with the ATP enzyme and D-Luciferin to produce light. Following this, the ADP enzyme was added to determine the ADP signal. The ADP/ATP ratio was quantified by luminescence with a BioTek Synergy HTX microplate reader.

NAD^+/NADH Quantities

NAD^+ and NADH quantification was determined using the NAD^+/NADH Quantification Kit (Sigma-Aldrich, MO, USA; MAK037) per manufacturer's directions. Cells were washed with pre-warmed phosphate-buffered saline (PBS) and incubated with pre-warmed serum-free DMEM with 1% P/S for 2 hours. Cells were then treated with pre-warmed treatment media containing vehicle, 20μM β-GPA, 500μM AICAR, or 10μM carbachol for 15 minutes. The cells were collected and pelleted in PBS and extracted with 400μL NADH/NAD^+ extraction buffer. The samples were deproteinized by spinning through a 10-kDa cut-off spin filter at 13,200 rpm for 15 minutes before the assay. For the detection of NADH only, NAD^+ was decomposed by heating 125μL of the samples to 60ºC for 30 minutes and cooling on ice. Samples were transferred to a 96-well plate and mixed with NAD^+ cycling buffer, NAD^+ cycling enzyme mix, and NADH developer. The plate was incubated at room temperature with minimum exposure to light for 4 hours. NAD^+ and NADH were quantified using absorbance measurement at 450 nm in

a BioTek Synergy HTX microplate reader. NAD^+ and NADH measurements were normalized to protein concentration (mg/mL), which was measured via Bradford assay (Bradford reagent: AMRESCO, Inc., Solon, OH).

Statistical Analyses

Statistical analyses were performed using IBM (Armonk, NY) SPSS Statistics 26. All values are expressed as mean ± SE. One-way ANOVA was used to examine differences between treatment groups; significance was set at $p < 0.05$.

CHAPTER 4

RESULTS

Changes in phospho-AMPK Levels

AMPK is known as the cellular energy sensor and regulator of metabolic pathways that affect energy expenditure and the flux of metabolism. Therefore, to explore whether β-GPA, AICAR, and carbachol directly affect AMPK in FL83B liver-like cells, western blot was used to determine changes in phospho-AMPK (p-AMPK) levels. Results are shown in Figure 2 and Figure 3 and expressed as p-AMPK/β-actin. There were no significant treatment effects on p-AMPK levels (vehicle: 1.03 ± 0.41, AICAR: 2.53 ± 1.30, β-GPA: 1.32 ± 0.17, and carbachol: 1.07 ± 0.27). These results indicate that acute β-GPA, AICAR, and carbachol treatments do not have significant effects on p-AMPK levels in FL83B cells.

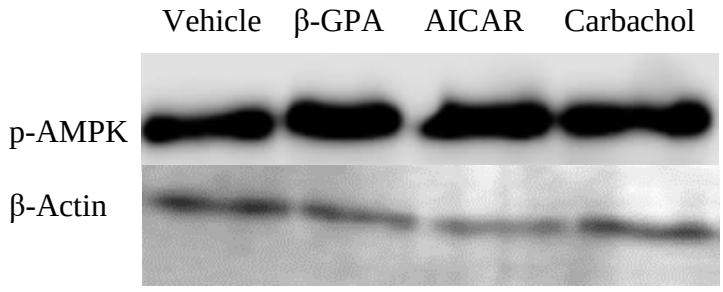


Figure 2. Western Blot of p-AMPK and β-Actin in FL83B Cells. Cells were treated with vehicle, 20μM β-GPA, 500μM AICAR, and 10μM carbachol for 15 minutes.

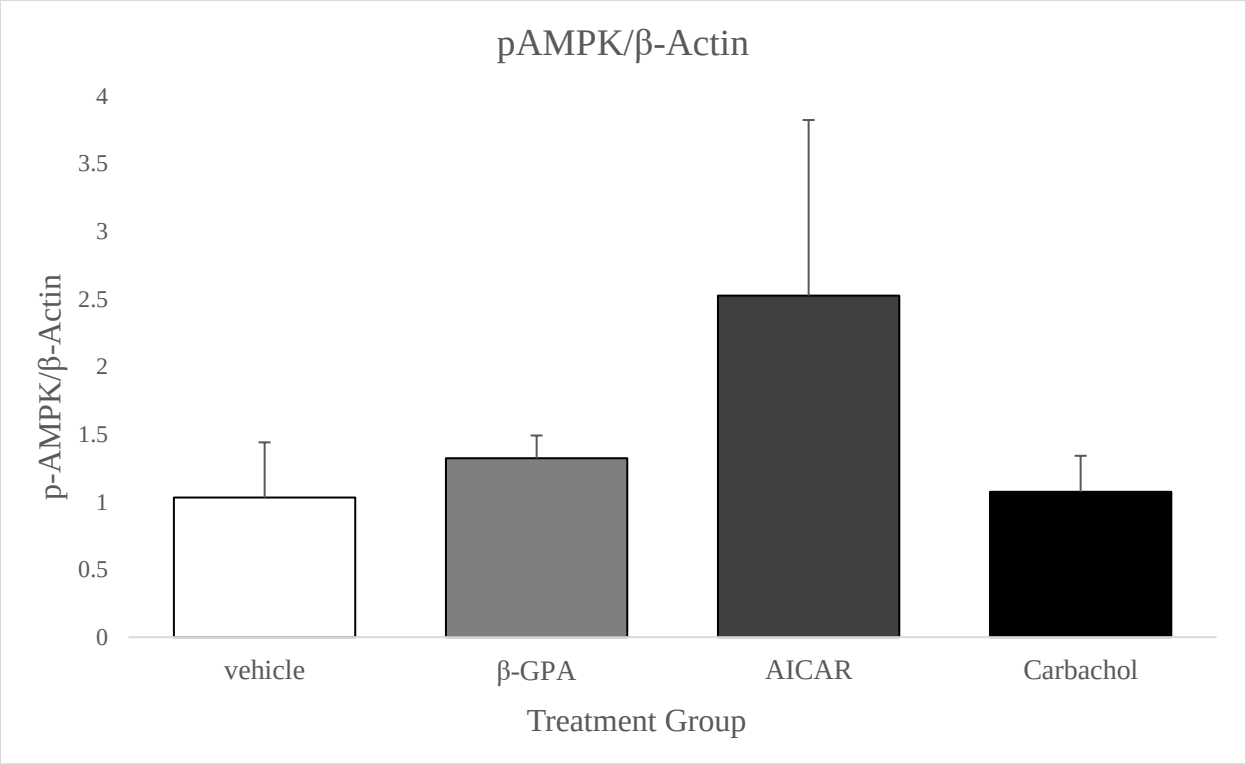


Figure 3. p-AMPK levels in FL83B cells. Cells were treated with vehicle, 20μM β-GPA, 500μM AICAR, and 10μM carbachol for 15 minutes. Changes in p-AMPK levels were determined by western blot then quantified by ImageJ. All values are expressed as mean ± SE.

ADP/ATP Ratio

In addition to p-AMPK activity, we next investigated whether β-GPA, AICAR, and carbachol treatments affect cellular energy levels through changes in concentrations of the intracellular energy indicators ADP and ATP. Results are shown in Figure 4. There was a significant difference between the β-GPA, AICAR, and carbachol treatment groups as determined by one-way ANOVA ($p = 0.01$). AICAR significantly increased the ADP:ATP ratio compared to vehicle (AICAR: 0.45 ± 0.07; vehicle: 0.28 ± 0.06, $p = 0.004$). There was a non-significant increase in carbachol-treated cells (carbachol: 0.41 ± 0.02; vehicle: 0.28 ± 0.06, p =

0.06) with no effect of β-GPA treatment (β-GPA: 0.23 ± 0.12; vehicle: 0.28 ± 0.06, p = 0.14). Taken together, AICAR is the most effective acute treatment to increase the ADP/ATP ratio in cells, indicating a lower energy status.

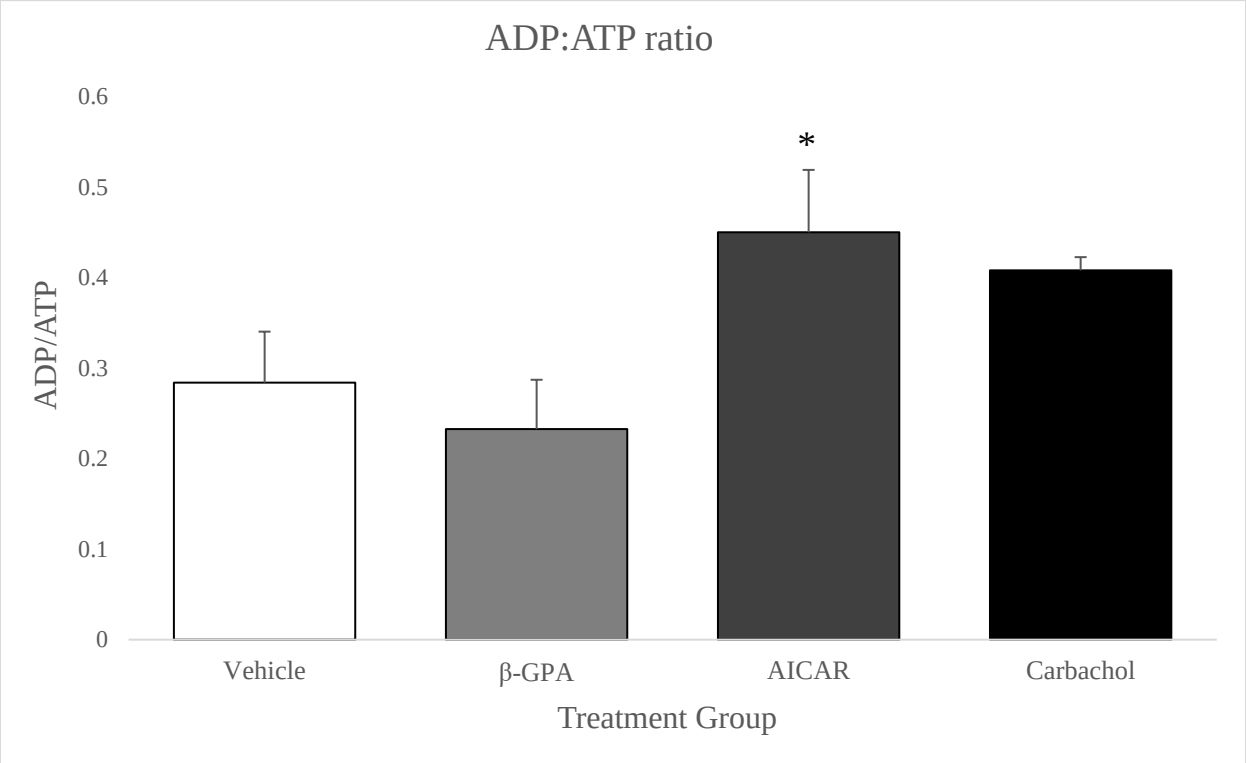


Figure 4. ADP:ATP ratios. FL83B cells were treated with vehicle, 20μM β-GPA, 500μM AICAR, and 10μM carbachol for 15 minutes. AICAR treatment caused a significant increase in the ADP:ATP ratio compared to vehicle whereas β-GPA and carbachol treatments did not significantly change. All values are expressed as means ± SE. $p < 0.05$. * = significant difference versus vehicle treatment.

NADH/NAD Ratio

Nicotinamide adenine dinucleotide (NAD^+), an electron carrier important to metabolism, is an indicator of metabolic flux. Increased levels of NADH, the reduced form of NAD^+, indicates increased catabolism. With a significant treatment effect of AICAR on cellular energy levels, we further explored whether this effect changes the redox state within liver-like cells as indicated by the NADH to total NAD^+ ratio. Results are shown in Figure 5. There were no significant treatment effects on NADH concentrations (vehicle: 0.15 ± 0.03, AICAR: 0.19 ± 0.02, β-GPA: 0.14 ± 0.02, carbachol: 0.14 ± 0.02). These results show that despite changing energy status of the cell, AICAR does not affect NADH concentrations in FL83B cells, and β-GPA and carbachol also do not affect NADH concentrations.

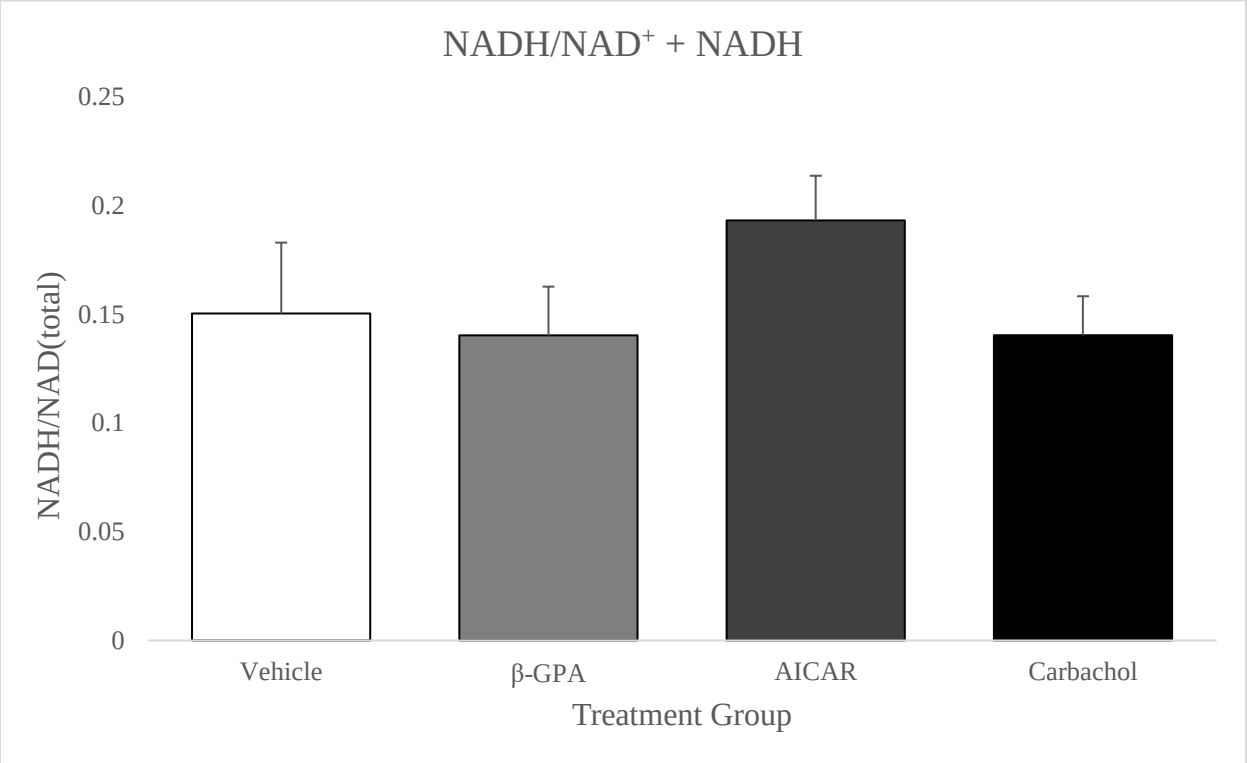
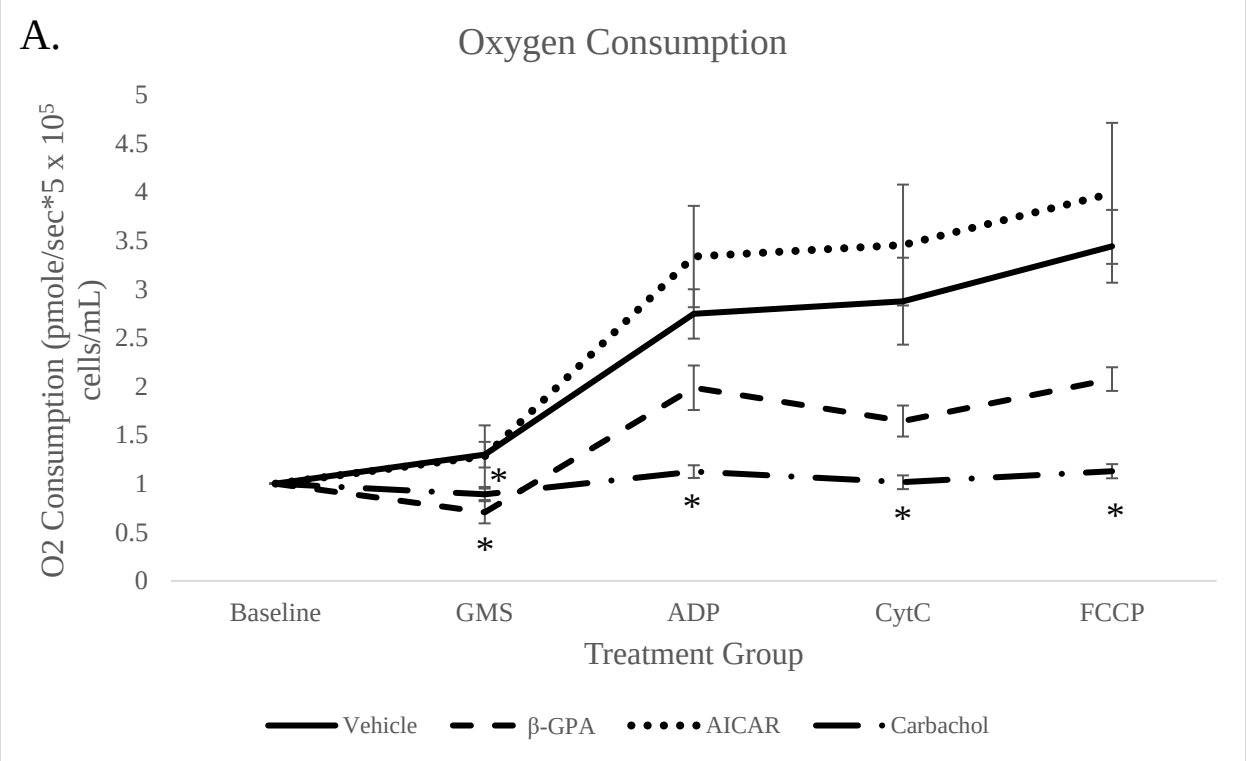


Figure 5. NADH/total NAD (NAD^+ + NADH) Ratios. FL83B cells were treated with vehicle, 20μM β-GPA, 500μM AICAR, and 10μM carbachol for 15 minutes. There were no significant treatment effects on NADH concentrations. All values are expressed as means ± SE.

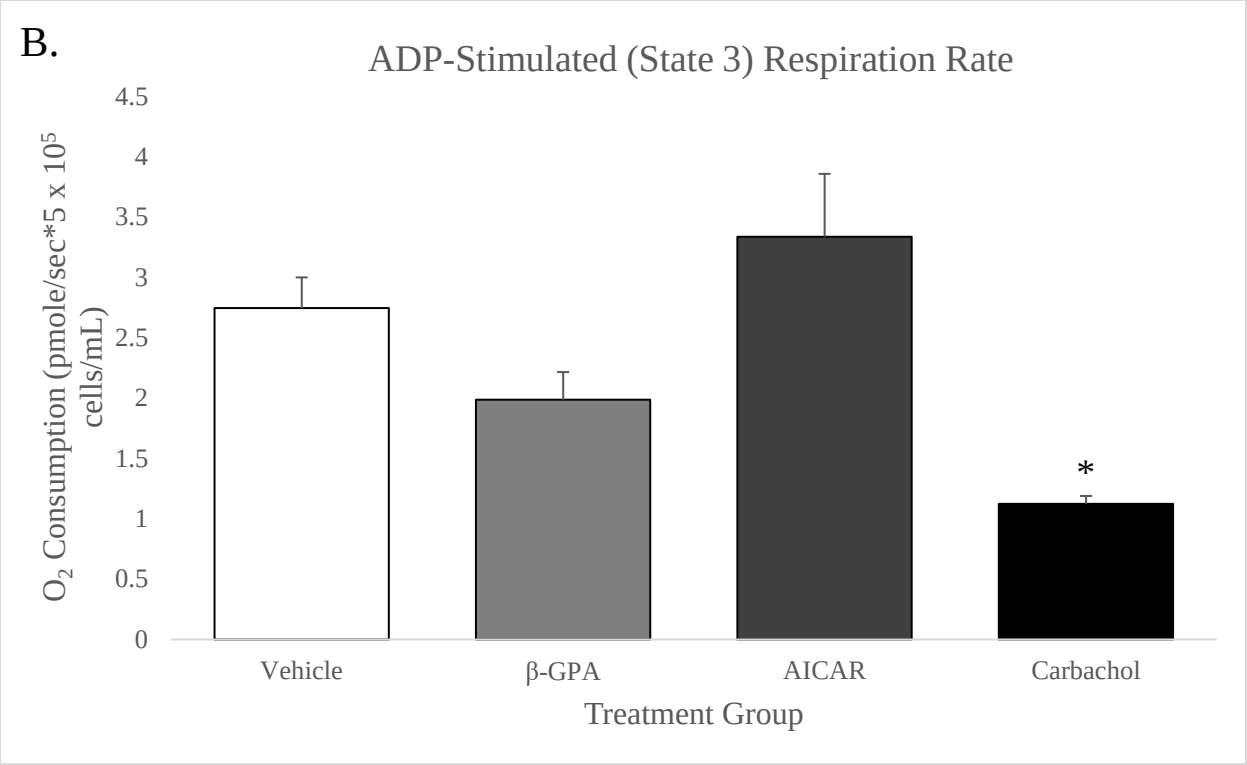
Oxygen Consumption

Oxygen consumption rates were used to determine the effects of acute β-GPA, AICAR, and carbachol treatments on mitochondrial state 3 (ADP-stimulated) respiration rate and maximal mitochondrial uncoupled (FCCP-stimulated) respiration rate, which are indicative of mitochondrial capacity and energy metabolism. Results are shown in Figure 6 and expressed as stimulated respiration rate divided by baseline respiration rate. After the addition of each substrate throughout the entire course of the oxygen consumption measurement, carbachol-treated cells had significantly lower respiration rates than vehicle treated cells (Glutamate, Malate, and Succinate (GMS): $p = 0.045$; ADP: $p = 0.001$; Cytochrome C: $p = 0.01$; FCCP: $p = 0.02$). β-GPA-treated cells had significantly lower respiration rates compared to vehicle after the addition of GMS (vehicle: 1.29 ± 0.13 pmole/sec*$5x10^5$ cells/mL; β-GPA: 0.71 ± 0.11 pmole/sec*$5x10^5$ cells/mL; $p = 0.02$) ADP-stimulated state 3 respiration rates increased from baseline 2.75 ± 0.25 pmole/sec*$5x10^5$ cells/mL for vehicle, 1.99 ± 0.23 pmole/sec*$5x10^5$ cells/mL for β-GPA, 3.34 ± 0.52 pmole/sec*$5x10^5$ cells/mL for AICAR, and 1.12 ± 0.07 pmole/sec*$5x10^5$ cells/mL for carbachol. State 3 respiration was significantly lower for carbachol-treated cells compared to vehicle-treated cells ($p = 0.001$) with no significant change for β-GPA or AICAR-treated cells. Uncoupled respiration rates increased 3.44 ± 0.37 pmole/sec*$5x10^5$ cells/mL for vehicle, 2.08 ± 0.12 pmole/sec*$5x10^5$ cells/mL for β-GPA, 3.99 ±

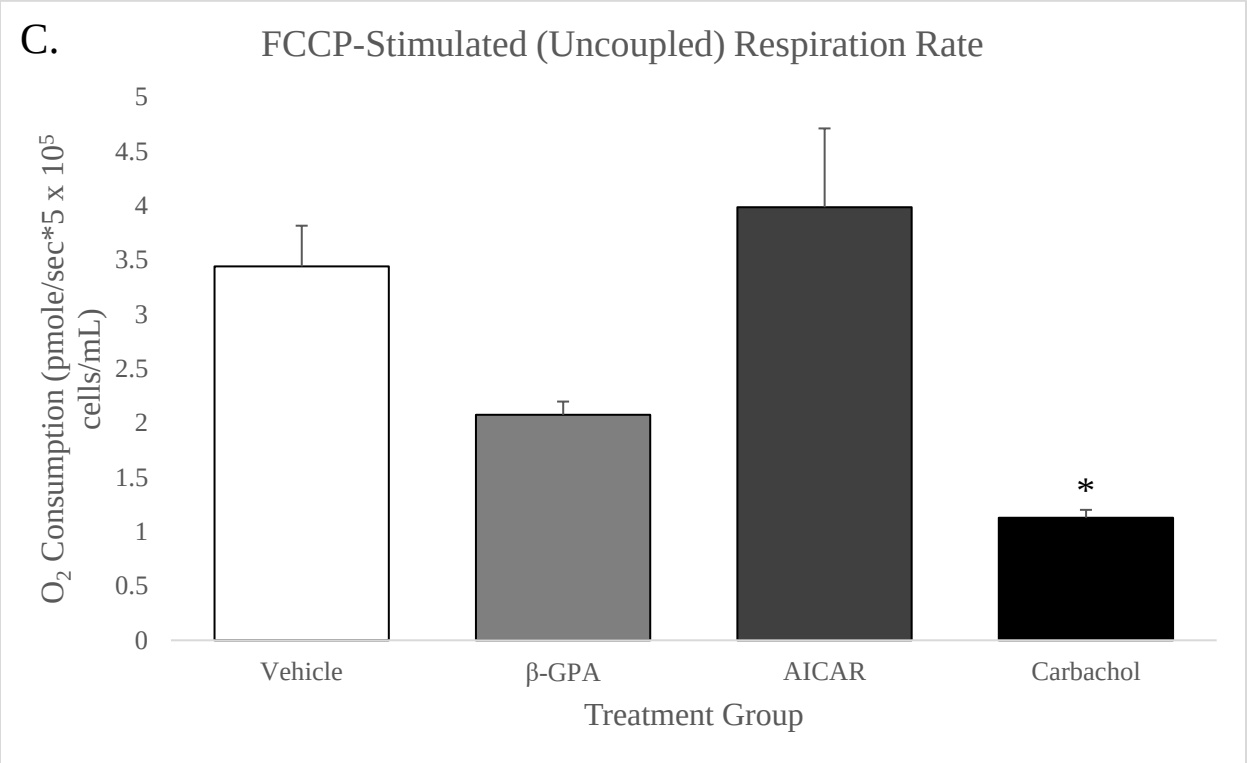
0.73 pmole/sec*5x10^5 cells/mL for AICAR, and 1.13 ± 0.07 pmole/sec*5x10^5 cells/mL for carbachol. Similar to the state 3 respiration rate, FCCP-stimulated uncoupled respiration rate was significantly lower for carbachol-treated cells compared to vehicle-treated cells ($p = 0.02$), with no significant change for β-GPA or AICAR-treated cells. The state 3 respiration rate:uncoupled respiration rate ratio was significantly higher compared to vehicle for both β-GPA and carbachol (vehicle: 0.79 ± 0.04; β-GPA: 1.03 ± 0.02, $p = 0.007$; carbachol: 1.01 ± 0.02, $p = 0.01$). Taken together, these results suggest that carbachol has a significant acute negative effect, β-GPA and AICAR have no effect on both state 3 and uncoupled respiration, but β-GPA and carbachol have significantly higher state 3 respiration:uncoupled respiration rate ratios.



B.


ADP-Stimulated (State 3) Respiration Rate
O2 Consumption (pmole/sec*5 x 10^5 cells/mL)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Vehicle
β-GPA
AICAR
Carbachol
Treatment Group

C.


FCCP-Stimulated (Uncoupled) Respiration Rate
O2 Consumption (pmole/sec*5 x 10^5 cells/mL)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Vehicle
β-GPA
AICAR
Carbachol
Treatment Group

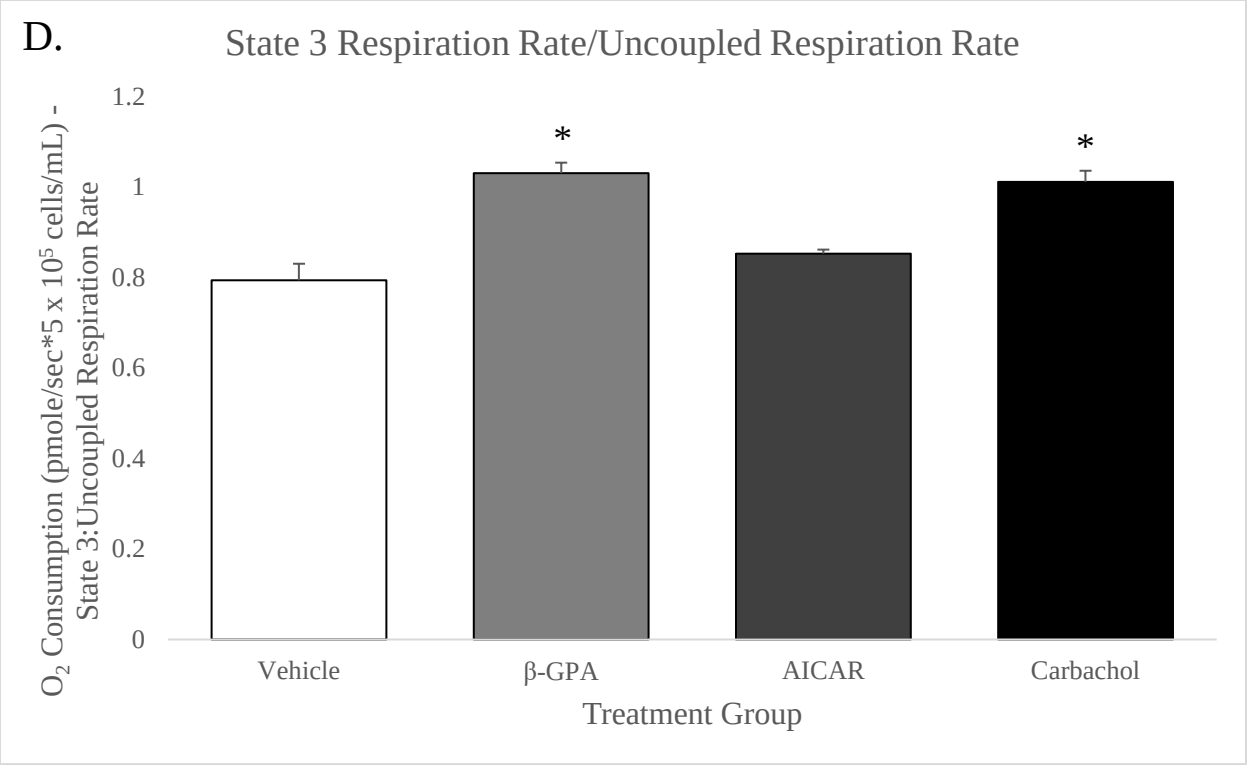
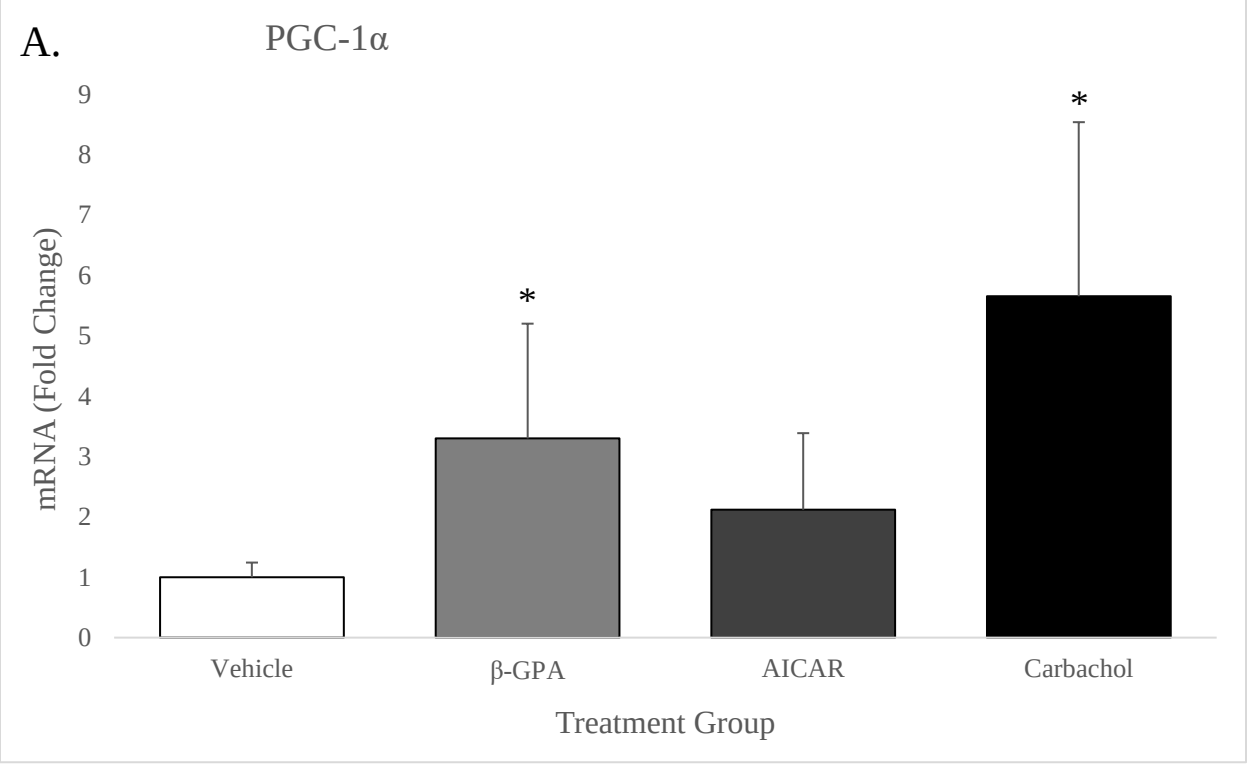


Figure 6. Oxygen Consumption Rates for Total (A), State 3 (B) and Uncoupled (C) Respiration and ADP/FCCP Rate (D). FL83B cells were treated with vehicle, 20µM β-GPA, 500µM AICAR, and 10µM carbachol for 15 minutes before addition of substrates. GMS = Glutamate, Malate, and Succinate; ADP = Adenosine Diphosphate; CytC = Cytochrome C; FCCP = Carbonyl cyanide-4-trifluoromethoxyphenylhydrazone. All values are expressed as means ± SE. $p < 0.05$. * = significant difference versus vehicle treatment.

Changes in PGC-1α and Perilipin-2 mRNA Expression

Quantitative real-time PCR was used to investigate the effects of β-GPA, AICAR, and carbachol treatments on mRNA expression of PGC-1α and perilipin-2. PGC-1α is a transcriptional coactivator that promotes mitochondrial biogenesis to increase capacity for oxidative metabolism, and perilipin-2 is a lipid-droplet coating protein that indicates fatty acid

turnover in cells. Results are shown in Figure 7 and indicate that chronic treatment (16 hours) results in significant increases in metabolic remodeling. One-way ANOVA indicated significant treatment effects both for PGC-1α ($p = 0.044$) and perilipin-2 ($p = 0.045$) expression. PGC-1α mRNA expression following β-GPA treatment was 3.30 ± 0.24-fold ($p = 0.02$), AICAR was 2.12 ± 1.90-fold ($p = 0.08$), and carbachol was 5.66 ± 2.88-fold ($p = 0.002$). For perilipin-2, β-GPA treatment increased mRNA expression 2.38 ± 0.33-fold ($p = 0.01$), AICAR 1.39 ± 1.07-fold ($p = 0.23$), and carbachol 3.29 ± 1.53-fold ($p = 0.004$). Taken together, these results show that β-GPA and carbachol, but not AICAR, are effective chronic treatments to significantly increase the expression of two genes associated with oxidative metabolism, PGC-1α and perilipin-2, in FL83B hepatocytes.



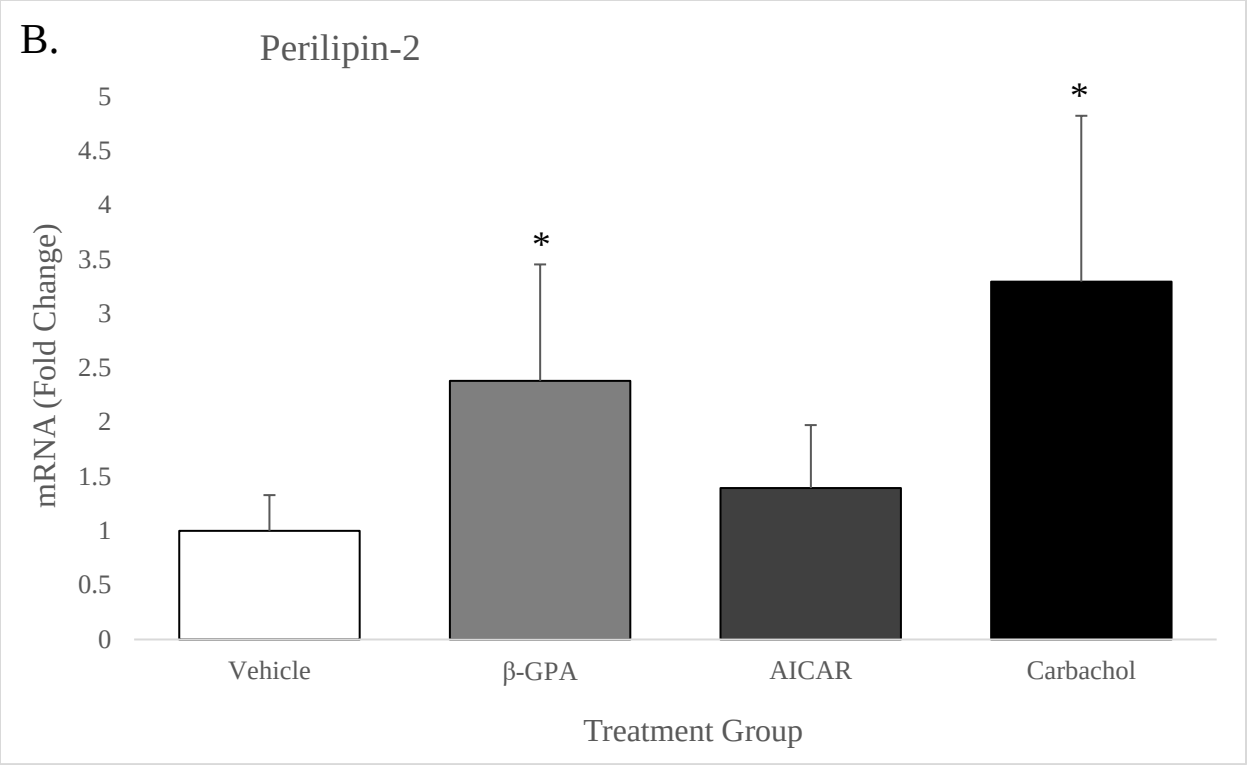


Figure 7. mRNA Expression of PGC-1α (A) and Perilipin-2 (B). FL83B cells were treated with vehicle, 20μM β-GPA, 500μM AICAR, 10μM carbachol for 16 hours. All values are expressed as means ± SE. $p < 0.05$. * = significant difference versus vehicle treatment.

CHAPTER 5

DISCUSSION

Cell culture is a valuable tool in nutrition research; however, when studying metabolic pathways at the basic level, the low rate of energy expenditure can pose limitations since the rate of metabolic flux does not compare to that of living organisms. Living organisms must respond and adapt to their environment, hormonal and temperature changes, and nutrient availability. On the other hand, cells in culture are maintained at a constant temperature with a constant supply of nutrients in a stable, unchanging environment. Because of this, cells in culture have relatively modest changes in energy expenditure and metabolic demand, while living organisms have increased energy expenditure and metabolic demand to maintain homeostasis. Therefore, we sought to determine if a pharmacological treatment could increase the flux of metabolism above baseline in cell culture. Three treatments, β-GPA, AICAR, and carbachol, have been shown to increase markers of oxidative metabolism and metabolic flux. β-GPA is a creatine analog that inhibits the creatine kinase reaction to alter cellular energy and has been shown to increase mitochondrial content, which increases oxidative capacity [31]. AICAR is an AMP analog and AMPK agonist that can increase fatty acid β-oxidation [20, 21]. Carbachol, an acetylcholine analog and muscarinic receptor agonist, affects glucose metabolism and can mediate metabolic changes [14, 33].

We hypothesized that energy expenditure and oxidative metabolism can be increased *in vitro* with the use of β-GPA, AICAR, or carbachol. However, this study demonstrated that these treatments may not be effective, as they produced little or no effect on increasing the flux of

metabolism *in vitro*. We treated FL83B cells with 20μM β-GPA, 500μM AICAR, or 10μM carbachol and measured p-AMPK activity, ADP/ATP and NADH/NAD^+ ratios, oxygen consumption, and changes in mRNA expression of PGC-1α and perilipin-2. The results of this study indicate that while acute (15-minute) AICAR treatment significantly increased the ADP/ATP ratio, its impact was modest and did not translate into chronic changes in gene expression. Conversely, acute β-GPA and carbachol treatments did not cause increased metabolic activity, but chronically, both β-GPA and carbachol increased PGC-1α and perilipin-2 expression.

We began by investigating the effects of these treatments on AMPK activity. AMPK is known as the cellular energy sensor and is phosphorylated and activated as adenosine monophosphate (AMP) concentrations increase and ATP concentrations decrease. Once activated, AMPK stimulates catabolic pathways, such as fatty acid and glucose oxidation, and inhibits anabolic pathways, such as lipogenesis and gluconeogenesis. AICAR has been shown to activate AMPK [15, 16]. Interestingly, our results showed no effect of AICAR treatment, and these contrasting findings could be a result of differences in concentrations or times used or methods that include AICAR treatment with other substrates such as AMP [15, 87]. Furthermore, differences in AMPK activation could be due to liver-like cells in culture having lower energy expenditure and energy demands than cells or tissues isolated from rats or mice.

To answer our question of which treatment causes a change in cellular energy status, we found that AICAR significantly increases the ADP/ATP ratio, indicating a lower cellular energy level, while β-GPA and carbachol have no effect. It is likely that this change in the ADP/ATP ratio, though significant, is not robust enough to affect p-AMPK levels given that our results demonstrate no significant change in p-AMPK levels. Further, this change in energy status does

not affect cellular redox state, which is associated with oxidative metabolism and fatty acid β-oxidation [88, 89]. Oxygen consumption rates are also not significantly changed by AICAR treatment, which indicates no change in mitochondrial respiration and activity.

Interestingly, despite no impact on metabolic markers, acute carbachol treatment has a significant negative effect on respiration rates, and β-GPA treatment resulted in a non-significant decrease in mitochondrial respiration. This suggests that carbachol could be affecting mitochondrial complexes I and II and subsequent ATP production. Although we were trying to increase metabolic activity, carbachol and potentially β-GPA were suppressing oxygen consumption, which indicates metabolic inefficiency. The mechanisms by which carbachol and β-GPA affect oxygen consumption are unclear from this study and warrant future research. However, we suspect that these treatments are producing changes that inhibit metabolic respiration, which leads to long-term metabolic adaptations, such as increased mitochondrial biogenesis. This idea is supported by our gene expression results.

Gene expression of PGC-1α, which is associated with mitochondrial biogenesis and increased fatty acid β-oxidation, and perilipin-2, which is associated with lipid droplet storage and turnover in cells, was significantly increased following β-GPA and carbachol treatments but not AICAR. This correlates with the results from acute (15-minute) treatments. For AICAR, unchanged oxygen consumption rates, p-AMPK levels, and redox state suggest that metabolic demand remained low, and because metabolism was unchanged, we expected there were not any longer-term cellular adaptations, such as increasing PGC-1α and perilipin-2 mRNA expression. However, we consistently observed increased expression under β-GPA and carbachol conditions, but we do not attribute their increases to altered metabolic flux. The oxygen consumption experiments showed acute negative results for carbachol and non-significant

decreases with β-GPA. It is possible that these acute treatments are causing metabolic inefficiency, based on the respiration/uncoupled respiration data. Further, the significantly higher state 3 respiration:uncoupled respiration rate observed with both β-GPA and carbachol indicate that uncoupling is occurring with these treatments. Because these treatments are resulting in changes that inhibit mitochondrial respiration, this could be producing longer-term adaptations, such as increased mitochondrial biogenesis, which correlates with the observed increase in PGC-1α expression.

To our knowledge, this is the first study to compare the effects of β-GPA, AICAR, and carbachol treatments on markers of energy levels and metabolic flux in FL83B cell culture, but it is not without limitations. This study used a fixed time and concentration for each treatment. Using a time-course design and/or various concentrations could uncover greater changes. Moreover, the oxygen consumption experiments were limited in power; running more trials would have increased the power and could potentially lead to greater or more significant changes. Additionally, as an *in vitro* and hypothesis-generating project, there is a higher chance for a type I statistical error. However, the results of this study show that these treatments do not change metabolic demand in cell culture. Future directions should explore other acute treatments, as there is still a need to develop a method to increase energy expenditure *in vitro*.

CHAPTER 6

CONCLUSION

To conclude, this proof-of-concept study showed that β-GPA, AICAR, and carbachol treatments are not effective treatments for increasing energy expenditure or metabolic demand in hepatocytes *in vitro*. Despite an acute effect of AICAR on ADP levels, metabolic flux remained largely unchanged. Additionally, the effect of β-GPA and carbachol on PGC-1α and perilipin-2 gene expression is likely not indicative of increased metabolic flux given the acute metabolic responses to these treatments. However, β-GPA and carbachol treatments could potentially be decreasing metabolic efficiency to result in these long-term adaptations. The results of this study warrant further research to investigate mechanisms behind the effects of β-GPA and carbachol on oxygen consumption and PGC-1α and perilipin-2 expression as well as other methods that can be used to increase metabolic flux *in vitro*. Developing a method to increase metabolic flux can be a beneficial tool for studying metabolism and targeting metabolic flexibility to improve liver health.

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