A single dose of metformin improves whole body insulin sensitivity and alters cellular redox state in skeletal muscle of Zucker fa/fa rats

by

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Energy balance is considered a fundamental requirement of life forms from single cell organisms to higher mammals such as humans. Unfortunately, our species has also discovered the detrimental metabolic responses to excess dietary intake: obesity and the accompanying pathologies collectively known as the metabolic syndrome. Central to the metabolic syndrome is insulin resistance, defined as a relative failure of insulin to stimulate glucose transport in peripheral tissues such as skeletal muscle. It is generally accepted that prolonged insulin resistance often results in the onset of type 2 diabetes, which is one of the most common diseases in the world. Current treatment for type 2 diabetes generally begins with dimethylbiguanide, an insulin sensitizing drug also known as metformin. In the last 10 years, scientific discovery has identified mitochondrial function as a key player in a variety of metabolic diseases, including insulin resistance and type 2 diabetes. As such, a variety of investigations have been performed in an attempt to indentify mechanisms by which altered mitochondrial function or physiology may contribute to the pathogenesis of these diseases. Recent evidence from our laboratory indicates that mitochondria derived oxidant (mROS) generation is a key player in the mitochondrial regulation of insulin sensitivity *in vivo*. Additionally, recent evidence has demonstrated that acute metformin treatment *in vitro* decreases liver mROS, and that chronic

metformin treatment *in vivo* decreases skeletal muscle mROS concurrent with improvements in whole body glucose tolerance. Together, this evidence indicates that metformin may alter peripheral insulin sensitivity by decreasing the elevated mROS associated with insulin resistance in the obese population. Therefore, the purpose of the current study was to investigate the effects of a single oral dose of metformin on whole body glucose tolerance and mROS in red and white gastrocnemius of Zucker fa/fa rats, a genetically obese animal model. A single oral dose of metformin resulted in improved whole body glucose tolerance compared to controls independent of alterations in serum insulin. Cellular redox state was significantly more oxidized in animals treated with glucose or metformin alone compared to controls or animals which received both treatments. Succinate and palmitoylcarnitine/malate induced mROS was not altered by glucose and/or metformin in red or white gastrocnemius. Mitochondrial respiration with pyruvate/malate or palmitoylcarnitine/malate was unchanged in response to glucose and/or metformin treatment in red or white gastrocnemius. Akt phosphorylation was significantly elevated in both red and white gastrocnemius in response to glucose or metformin alone, but no additive effect was observed when administered simultaneously, indicating that metformin may act as an insulin mimetic *in vivo*. AMPK phosphorylation was not elevated in response to metformin treatment in either tissue, which suggests that metformin may act through AMPK-independent mechanisms in skeletal muscle *in vivo*. The results of this study demonstrate that a single oral dose of metformin can improve whole body glucose tolerance independent of changes in mitochondrial respiration, mROS, or altered AMPK signaling in red and white gastrocnemius of Zucker fa/fa rats, but may be associated with altered cellular redox state.

A single dose of metformin improves whole body insulin sensitivity and alters cellular redox state in skeletal muscle of obese Zucker fa/fa rats

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LIST OF SYMBOLS AND ABBREVIATIONS

ADP - adenosine diphosphate

Akt - protein kinase B

AMP - adenosine monophosphate

AMPK - adenosine monophosphate-activated protein kinase

AS-160 - Akt substrate of 160 kilodaltons

ATP - adenosine triphosphate

BaO - barium orthovanadate

BCA - bicinchoninic acid

Bleb - blebistatin

Buffer X - relaxing/permeabilization buffer

Buffer Z - assay buffer

CoA - coenzyme A

CPT - carnitine palmitoyl transferase

DAG - Diacylglycerol

EDTA - ethylenediaminetetraacetic acid

EGTA - ethylene glycol tetraacetic acid

ELISA - enzyme linked immunosorbent assay

ETS - mitochondrial electron transport system (a.k.a. electron transport chain)

FAO - fatty acid oxidation

FFA - free fatty acid

GLUT - glucose transporter protein

GSH - glutathione

GSSG - glutathione disulfide

H_2O_2 - Hydrogen Peroxide

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HOMA - homeostatic model assessment

IMS - mitochondrial intermembrane space

IR - insulin receptor

IRS - Insulin receptor substrate

JNK - c-Jun NH_2-terminal kinase

K_m - half-maximal rate of enzyme activity

K-MES - potassium- 2-(*N*-morpholino)ethanesulfonic acid

LCACoA - long chain acyl coenzyme A

MAPK - mitogen-activated protein kinase

mM - millimolar, $1 \cdot 10^{-3}$ molar concentration

μM - micromolar, 10^{-6} molar concentration

mOsm - milli-osmolarity, $1 \cdot 10^{3}$ moles of solute particles per liter of solution

mROS - mitochondrial oxidant generation

μU - microunits, 1·10-6 SI units of a substance

NaF - sodium fluoride

NaO - sodium orthovanadate

NaPPi - sodium pyrophosphate

O_2 - molecular oxygen (a.k.a. diatomic oxygen)

O_2^- – superoxide (negatively charged oxygen radical)

PCr - phosphocreatine

PDK - phosphinositide-dependent kinase

PGC - peroxisome proliferator-activated receptor γ co-activator

PI-3K - phosphatidylinositol-3 kinase

PKC - Protein Kinase C

PPAR - peroxisome proliferator-activated receptor

PTP1B - protein tyrosine phosphatase isoform 1B

PVDF - polyvinylidene fluoride

RNA - ribonucleic acid

SDH - succinate dehydrogenase

SOD - superoxide dismutase

T2DM - non-insulin dependent diabetes mellitus (aka. Type 2 Diabetes)

TCA - tricarboxylic

TNF - tumor necrosis factor

TORC - mammalian target of rapamycin Rictor complex

TSC - tuberous sclerosis factor

UCP - uncoupling protein

UCR - uncoupling control ratio

V_{max} - maximum velocity of an enzyme

CHAPTER 1: REVIEW OF LITERATURE

Introduction

The prevalence of metabolic disease has been increasing to near pandemic rates in the past few decades. A primary component of the metabolic syndrome is insulin resistance, which is associated with a myriad of associated pathologies, which include obesity and type II diabetes mellitus (T2DM). Dimethylbiguanide, better known as metformin, has been used since the 1930's in the treatment of T2DM. Although metformin has been extremely effective and perhaps the most commonly prescribed anti-diabetic drug in the last 50 years, the potential mechanisms of action for metformin are still largely unknown. Recently obtained data in our laboratory has demonstrated a role for mitochondrial derived hydrogen peroxide (H_2O_2) in the pathogenesis of insulin resistance. Our laboratory proposes that elevated mitochondrial superoxide ($O_2^{-}\cdot$) production and subsequent H_2O_2 emission (mH_2O_2) can induce a shift in the cellular redox environment to a more oxidized state, the result of which appears to be a major contributor to insulin resistance. By decreasing mH_2O_2 in rat skeletal muscle mitochondria using pharmacological and transgenic manipulations, Anderson et al. (3) demonstrated that maintaining a reduced cellular redox environment can prevent high fat diet induced insulin resistance in high fat fed rats and mice. Furthermore, our laboratory has recently demonstrated that the anti-diabetic action of metformin treatment is associated with decreased mitochondrial oxidant generation, specifically from Complex I of the electron transport system (ETS) (9). It has been shown previously that metformin is a mild inhibitor of complex I of the ETS and metformin appears to decrease reverse electron flow through Complex I, which is a major

contributor of mH_2O_2 (39). The purpose of this project was therefore to determine whether treatment with a single oral dose of metformin improves insulin sensitivity in the Zucker fa/fa rat, a genetic model of obesity and insulin resistance.

Obesity and Diabetes

Obesity has become an increasingly important health issue in recent years, particularly when considering the increasing prevalence of other metabolic syndrome related pathologies that accompany obesity. Obesity results in an increased risk of developing non-insulin dependent diabetes mellitus (T2DM). The link between obesity and T2DM has been thought to be largely due to the development and progression of skeletal muscle insulin resistance over the course of years (10). Simply put, insulin resistance is defined as a relative failure of insulin to induce glucose transport in insulin sensitive tissues, principally skeletal muscle and adipose tissue. Skeletal muscle insulin resistance *in vivo* appears to be associated with reduced capacity for intramuscular lipid oxidation (reviewed by (32, 52)); however, the specific molecular mechanisms relevant to *in vivo* insulin resistance are incompletely understood. Previous work has shown that obesity is associated with an increase in plasma and intramuscular lipid accumulation, both of which have been implicated as major contributors to the development and progression of skeletal muscle insulin resistance (41, 49).

Insulin sensitivity is negatively correlated with plasma free fatty acids (FFA) in subjects with T2DM, and the extent to which insulin suppresses plasma FFA is thought to be a strong indicator of the severity of T2DM (41). Further evidence to support the contribution of obesity to insulin resistance was provided by Amati et al. (2) who found that, regardless of age, insulin sensitivity was dependent on physical activity and obesity. Additionally, Williams et al. (78)

found that clinically significant weight loss caused a large, yet incomplete, increase in insulin sensitivity in subjects with T2DM. It is clear that based on the existing literature, obesity is a major contributor to insulin resistance and the pathogenesis of T2DM.

Skeletal muscle of obese individuals displays a number of distinct features, perhaps most importantly a decrease in mitochondrial functional capacity, which appears to result from a decrease in both respiratory capacity (11, 51, 59) and mitochondrial content (59). Obese individuals have also been shown to have lower fatty acid oxidation (FAO) at rest than lean individuals (45), and it has been suggested that this dysregulation in FAO is an essential factor contributing to insulin resistance in the obese state (40). These observations have led to the hypothesis that insulin resistance may stem from a lower capacity to oxidize lipids in skeletal muscle as a result of acquired or inherited mitochondrial dysfunction which, in turn, leads to the accumulation of lipid metabolites that disrupt the insulin signaling pathway (47).

Lipid Intermediates

The obese phenotype provides an indication of decreased mitochondrial function, but recent evidence suggests that an acute high-fat diet or lipid infusion can induce insulin resistance independent of mitochondrial dysfunction by causing an elevation in lipid intermediates, specifically two products of long chain fatty acyl-CoA (LCACoA): diacylglycerol (DAG) and ceramide (22, 44). LCACoA is the metabolically active form of triglyceride within the cell, and as such they are either sent to the mitochondria for β-oxidation or used as an intermediate for other lipid species such as DAG or ceramide. DAG can be generated in a number of ways, but the most important source is via *de novo* synthesis by esterification of 2 LCACoAs to glycerol-3-phosphate (74). DAG has been implicated as a contributor to lipid-induced insulin resistance indirectly based on correlations found between DAG and PKCθ in Goto-Kakizaki and Zucker

rats (5). Yu et al. (79) also found that lipid infusion caused a significant increase in DAG which was associated with PKCθ activity and increased IRS-1 Ser^{307} phosphorylation.

Ceramide represents another intracellular lipid metabolite implicated in the development of insulin resistance. Ceramide is a lipid metabolite generated in a similar fashion as DAG, and has been shown to directly activate PKC ζ, which suppresses Akt activity, and may contribute to insulin resistance (21). An important difference between the effects of ceramide versus DAG on insulin resistance was found by Chavez et al (14), who found that ceramide contributed to saturated fat induced insulin resistance, but not DAG. This does not nullify the potential role that DAG plays in lipid-induced insulin resistance, but is certainly noteworthy when considering the relative role of each. The link between insulin resistance and obesity very well may be in the generation of DAG and ceramide (35); however, there is growing evidence that also suggests that decreases in β-oxidation may also play a large role in the etiology of insulin resistance independent of intramuscular lipid accumulation (56).

Fatty Acid Oxidation

Evidence supporting the theory that limitations in β-oxidation contribute to the development of insulin resistance has been primarily focused on the enzyme carnitine palmitoyltransferase 1 (CPT-1). CPT-1 is an enzyme that is integral to β-oxidation as it increases fatty acid oxidation by increasing the uptake of long chain fatty acyl-CoA into the mitochondria. CPT-1 plays an important role in the relationship between fatty acid oxidation, lipid intermediate generation and insulin resistance as evidenced by Dobbins et al. (19), who found that prolonged inhibition of CPT-1 or a diet high in saturated fat promoted diacylglycerol accumulation and insulin resistance in rats. Further evidence of CPT-1 being integral to fatty acid oxidation was provided by Bruce et al. (12), who has shown that overexpressing CPT-1 in

skeletal muscle increases fatty acid oxidation, ameliorates lipid-induced insulin resistance in skeletal muscle, and does so without changes in mitochondrial capacity or function. These findings suggest that the capacity for fatty acid oxidation is a contributor to insulin resistance in skeletal muscle in both the obese and high fat fed state, although the potential impact of overexpressing CPT1 on mitochondrial inner membrane integrity and total energy turnover was not considered or tested.

Despite the popular theory of "mitochondrial dysfunction" as an underlying cause of insulin resistance (47), there is growing evidence indicating that a high-fat diet induces an initial increase in β-oxidation capacity and that loss of mitochondrial content only occurs after prolonged high fat intake (several months), well after the development of insulin resistance (31). Direct support of an increase in mitochondrial fatty acid oxidation resulting from a high fat diet was provided by Turner et al. (75), in which rats fed a high-fat diet for 5 or 20 weeks both showed an increase in palmitate oxidation compared to rats fed standard chow. This increase in palmitate oxidation was accompanied by increased peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) and uncoupling protein (UCP)-3 protein expression. Hancock et al (28) found that rats fed a high-fat diet for 4 weeks displayed significant increases in PGC-1α protein although PGC-1α mRNA expression decreased. The authors determined that the elevated FFA resulting from the high-fat diet caused a significant increase in peroxisome proliferator-activated receptor δ (PPARδ) mRNA expression, which increased PGC-1α protein expression. Insulin resistance still occurred in the muscle as indicated by reduced insulin-stimulated 2-DG transport assessed in vitro, clearly in the absence of any change in the mitochondria (28). In addition, Garcia-Roves et al. (23) observed an increase in PPARδ binding to the muscle carnitine palmitoyltransferase 1 (mCPT-1) promoter in epitrochlearis muscle after

4 weeks of elevated FFA. This was accompanied by a ~60% increase in palmitate oxidation, an increase in mCPT-1 mRNA, UCP-3 mRNA and mitochondrial TCA cycle enzyme activity. It is interesting to note that mitochondrial biogenesis occurred without any observed change in PGC1-α gene expression. Therefore, during a high-fat diet, it appears that fatty acid oxidation increases despite the development of insulin resistance.

Insulin Signaling

The insulin signaling pathway has long been implicated as the target of insulin resistance by obesity, high fat diet and lipid accumulation, and as such there are a number of targets in the insulin signaling pathway that are thought to be associated with insulin resistance. Some of the components of the insulin signaling pathway implicated in insulin resistance include: Insulin Receptor (IR), Insulin Receptor Substrate-1 (IRS-1), Phosphatidylinositol-3 Kinase (PI-3K), Protein Kinase B (Akt), and Akt substrate of 160 kDa (AS-160). In skeletal muscle, the insulin signaling cascade begins with insulin binding to the receptor on the cell membrane, which activates intrinsic tyrosine kinase activity of the IR which leads to auto-phosphorylation of the IR β-subunit and subsequent tyrosine phosphorylation of IRS-1. Tyrosine phosphorylation of IRS-1 recruits the p85 subunit of PI3K to the cell membrane (73). From there, PI3K phosphorylates phosphatidylinositol 3,4-diphosphate (PIP_2) to PIP_3, and the increase in PIP_3 recruits proteins containing pleckstrin homology (PH) domains, such as Akt and phosphinositide-dependent kinase 1 (PDK-1) to the cell membrane. When Akt reaches the cell membrane, it is phosphorylated by PDK-1 on Thr^{308} (70, 71) and the mTOR-Rictor complex 2 (TORC2) on Ser^{473} (33, 64). Once Akt is activated, AS160 is phosphorylated, which relieves the "brake" on GLUT4 translocation as evidenced by Sano et al. (63) who showed that when a mutant AS160 is deactivated, previous inhibition of GLUT4 translocation is alleviated. Evidence that FFA

accumulation is associated insulin resistance was provided by Griffin et al. (27), who found that performing a 5-hour lipid infusion increased plasma FFA in Sprague Dawley rats, and the increased plasma FFA was associated with significant decreases in insulin-stimulated PI3K activity and IRS-1 tyrosine phosphorylation, as well as decreased glucose uptake. Further supporting the obesity induced theory of insulin resistance, Standaert et al. (68) found that obese diabetic rhesus monkeys had significantly less IRS-1/PI3K activity as well as decreased insulin stimulated glucose uptake. Based on the current literature, considerable evidence exists to suggest that FFA accumulation may be at least one mechanism by which high lipid intake induces insulin resistance.

There is certainly evidence indicating that disruptions in the insulin signaling pathway caused by obesity and/or lipid accumulation contribute to insulin resistance; however, the extent that these disruptions contribute physiologically is still very controversial (22, 44). The reason for the controversy is because although there may be disruptions in the insulin signaling pathway, the response may be non-linear, and may not even physiologically inhibit glucose uptake (44). This thinking is based on a paper from Whitehead et al. (76) who showed that maximal insulin-stimulated glucose transport in adipocytes is achieved by activating only 10% and 20% of IRS-1 and Akt, respectively. Although this may not hold true for skeletal muscle, especially when considering the relative quantity of glucose taken up by skeletal muscle compared to adipose tissue, the question still remains whether inhibition of the insulin signaling pathway by high fat diet or lipid intermediates inhibits glucose uptake under physiological conditions.

Electron leak and Superoxide generation

The electron transport system is located on the mitochondrial inner membrane, which consists of four complexes, and is responsible for producing ATP with oxygen serving as the final electron acceptor to form H_2O. Although not pathological, during basal mitochondrial respiration a relatively small fraction of electrons (0.2-2%) leak from the ETS (13, 69). Electron leak occurs at either complex I or III of the electron transport system as $O_2^{-}\cdot$ and are converted to H_2O_2 by SOD in either the matrix or intermembrane space. H_2O_2 has an integral function in cell signaling through reversible cysteine oxidation (58). Although H_2O_2 is emitted at low levels during basal mitochondrial respiration, H_2O_2 emission can increase dramatically under a variety of both physiological and pathophysiological conditions (4, 59). When H_2O_2 emission is elevated, the redox state of the cell is altered due to the reversible oxidation of cysteine residues with specific proteins resulting in an overall shift in cellular redox environment to a more oxidized state (38).

Regulation of Cellular Redox state

Since the maintenance of the redox environment is clearly essential to the proper functioning of a cell, a number of therapies have been developed to attempt to prevent the production/accumulation of oxidants. The drawback to antioxidant therapies in general is that most antioxidants are water soluble and, therefore, do not reach a sufficient concentration in the mitochondrial matrix to effectively scavenge $O_2^{-}\cdot$.

Endogenously, cellular redox state is maintained primarily by the tri-peptide glutathione (GSH). The oxidation of two glutathione molecules by glutathione peroxidase yields glutathione disulfide (GSSG); GSSG can be converted back into two glutathione molecules by the enzyme glutathione reductase. GSH is found mainly (>98%) in the reduced form in the cytosol of

mammalian cells, with very little (<1%) found as GSSG (65). The ratio of GSSG to GSH is commonly used as an index of the redox state of the cell, with a greater ratio indicative of oxidative stress. Glutathione performs a variety of functions within a cell, from being the most abundant oxidant scavenger in the cytosol (50) to being an essential "on/off" switch for a number of cell signaling pathways through S-glutathionylation (17).

Although the electron transport system is located on the inner membrane of the mitochondria, $O_2^{-}\cdot$ is not released exclusively to the mitochondrial matrix. Whereas $O_2^{-}\cdot$ generated by complex I is released exclusively into the mitochondrial matrix, it has been recently shown that ~50% of $O_2^{-}\cdot$ produced by complex III is released into the matrix and ~50% released into the intermembrane space (53, 67). Recent work by Hu et al. (34) has shown the intermembrane space (IMS) is far more oxidizing than the mitochondrial matrix, and that the redox environment in the mitochondrial matrix and IMS are independently regulated by different isoforms of GSSG reductase. This information combined with SOD isoforms being location specific (16) and $O_2^{-}\cdot$ being highly membrane impermeable (43) ultimately demonstrates that separate compartmentalized redox environments are present within the mitochondria. Therefore, it is clear that antioxidant therapies need to be specific in their localization in order to reach the mitochondrial matrix to scavenge electrons.

Altered Insulin signaling by H_2O_2

Oxidation of proteins can be particularly detrimental when the redox potential of the cell is already compromised, which occurs when there is an abundance of H_2O_2 present in the cell. H_2O_2 emission has been shown to increase in skeletal and cardiac muscle as a result of high fat feeding, and results in a disruption of the redox status of the cell (3). A shift towards a more oxidative state in a cell causes disruption in numerous signaling pathways that rely on oxidation

as a key regulating mechanism. The two main forms of reversible protein modification are glutathionylation and cysteine oxidation.

Glutathionylation is a reaction that occurs when a cysteine residue on the tri-peptide glutathione (GSH) and a cysteine residue on a protein thiol are oxidized, and subsequently bind to form a mixed disulfide. There are a number of different ways in which GSH can interact with a protein thiol, such as thiol/disulfide exchange and thiol oxidation, depending on the oxidation state of the thiol and GSH. Protein S-glutathionylation occurs during basal conditions, suggesting a regulatory signaling function during physiological conditions (25). The primary targets of glutathionylation are sulfhydryls and disulfides on proteins in oxidation sensitive regions, which are regions that are very basic (low pKa) or overly exposed due to their 3-D structure. Beyond serving a regulatory role, it has been suggested that S-glutathionylation serves as a way of storing glutathione when it is sequestered as a result of binding to the protein thiol, as well as protecting protein thiols from non-reversible oxidation (48). Glutaredoxin plays a role in both S-glutathionylation and in protein deglutathionylation.

Cysteine is an amino acid that is particularly sensitive to oxidation, and is also a key component of numerous enzymatic reactions (58). Active-site cysteine acts as a switch for a large number of cell signaling proteins, and is also quite abundant in the ion channels and receptors in the plasma membrane of cells. Therefore, when the redox environment of the cell shifts to a more oxidized state, critical cysteine residues within specific key proteins can become glutathionylated or oxidized which can result in altered regulation of cell function.

A growing line of inquiry linking insulin resistance to a disrupted redox state is focused on the downstream targets of TNFα, such as p38 mitogen-activated protein kinase (p38-MAPK) and the c-Jun NH_2-terminal kinase (JNK). JNK is activated by a number of stimuli, including

inflammatory cytokines such as tumor necrosis factor-α (TNF-α). The activation of JNK has been shown to positively correlate with serine^{307} phosphorylation of IRS-1 (30), which inhibits tyrosine phosphorylation of IRS-1 and in turn prevents downstream phosphorylation of phosphatidylinositol 3-kinase (PI3K), ultimately resulting in insulin resistance. Further support implicating JNK as a negative regulator of IRS-1 tyrosine phosphorylation was provided by Lee et al. (46) which showed that a decrease in Ser^{307} phosphorylation and an increase in tyrosine phosphorylation in cells expressing a mutant IRS-1 lacking a JNK binding domain. The decreased Ser^{307} phosphorylation was directly related to increased insulin-stimulated tyrosine phosphorylation, Akt phosphorylation and increased glucose uptake. The prospect of JNK and upstream factors such as TNFα contributing to insulin resistance is a popular line of research currently.

PTP1B is an interesting target because it has been shown to have an essential function in insulin signaling by dephosphorylating tyrosine residues on both the IR and IRS-1, which negatively regulates insulin signaling. PTP1B is only active in the reduced (thiolate) form, but is highly susceptible to oxidation. The reversible oxidized form (suphenic acid) can be converted back to the active form by amination to sulphenyl-amide and subsequent reduction (18). During highly oxidizing conditions, PTP1B can be oxidized to sulphinic or sulphonic acid, which is irreversible. Barrett et al. (8) found that s-glutathionylation prevented further oxidation from sulphenic acid, and that glutathionylation was an important regulator of PTP1B function. Although PTP1B may be an appealing target for studying insulin resistance, Zabolotny et al. (80) found that mice overexpressing PTP1B were shown to be insulin resistant, thus indicating that the dephosphorylation of the β-subunit of IR caused by the active form is a much larger contributor to insulin resistance than the concurrent dephosphorylation of IRS-1. A possible

explanation for this may be that PTP1B was only shown to be a substrate for IRS-1 *in vitro* (26), so this response may not occur *in vivo*. Therefore, available evidence to date indicates that a more oxidized redox environment may contribute to insulin resistance through MAPKs but probably not through regulation of PTP1B, and may be partially regulated by glutathionylation via regulatory pathways incorporating cysteine residues, but no specific pathways have been elucidated.

Treatment of T2DM with Metformin

Biguanides have been used as medicine since medieval Europe in the form of French lilac. Two specific biguanides, phenformin and metformin, were finally isolated and synthesized in the 1930's. Phenformin is no longer in use due to the propensity for phenformin to induce lactic acidosis, but metformin has been in use in most of Europe since the 1950's and in the United States since the 1980's (6). Dimethylbiguanide, better known as metformin, is one of the most widely prescribed pharmacological agents for T2DM. Clinically, it is well established that metformin improves fasting glucose levels by decreasing hepatic glucose output and improving glucose tolerance by increasing insulin stimulated peripheral glucose uptake (reviewed in (7)). Although the clinical utility of metformin treatment is well established, the physiological mechanisms by which it acts are incompletely understood. There are two predominantly interwoven theories regarding the physiological mechanism of metformin action: altered electron flow through Complex I of the mitochondrial electron transport system (ETS) and activation of adenosine monophosphate activated protein kinase (AMPK). Understanding the link between altered Complex I function and AMPK activation in the context of diabetes will be useful to elucidate the *in vivo* nature of metformin.

Cellular metabolism is very tightly controlled, with small alterations in energy state sometimes resulting in dramatic physiological changes. An excellent example of the tight regulation of metabolism is the equilibrium state of the family of adenosine phosphates (ATP/ADP/AMP). Since mitochondria are the primary source of ATP production in mammalian cells, mild alterations in the ETS of the mitochondria can result in global changes in cellular energy state (24). Furthermore, since [ADP] and [AMP] are sustained at extremely low concentrations, very small absolute changes in either molecule can result in large relative changes. Metformin has been shown to alter Complex I of the ETS in two ways: decreasing forward electron flow through Complex I which can result in decreased mitochondrial respiration and decreased [ATP], or decreasing reverse electron flow through Complex I which can result in decreased mitochondrial oxidant emission (9). It has been shown recently in our lab that incubating rat red gastrocnemius muscle in different concentrations of metformin has concentration dependent effects on electron flow through Complex I (39). Particularly, a ~100-fold greater concentration of metformin was required to inhibit forward electron flow (evidenced by decreased mitochondrial O_2 consumption) than was needed to inhibit reverse electron flow (evidenced by lower succinate supported H_2O_2 emission). This data suggests that since reverse electron flow was altered at a much lower concentration of metformin, perhaps inhibition of reverse electron flow is a more relevant *in vivo* mechanism of action for metformin than the inhibition of forward electron flow.

AMPK, a master cellular energy sensor (reviewed by (29)), can modulate metabolic pathways in response to altered energy states (i.e. increased [AMP]). AMPK is present in a wide variety of cell types, including skeletal muscle and liver, and acts as a master metabolic switch in response to alterations in cellular energy state. For example, AMPK activity is increased in

response to energy deficit (i.e. fasting conditions), which inhibits mTOR signaling by activating tuberous sclerosis factor (TSC) 1/2 and results in decreased protein synthesis (36). The more general result of an increase in AMPK activity is a decrease in the activity of energy consuming pathways (i.e. protein synthesis) and an increase in the activity of energy producing pathways (i.e. β-oxidation and glycolysis).

An increase in AMPK phosphorylation in response to 4 and 10 weeks of oral metformin treatment has been observed in type 2 diabetic human vastus lateralis, and was associated with improved skeletal muscle glucose transport in those subjects (54). Our lab has recently demonstrated that 4 weeks of oral metformin treatment induces improvements in whole body glucose tolerance in obese insulin resistant Zucker fa/fa rats (39). Interestingly, Suwa et al, (72) have shown that AMPK phosphorylation can be significantly elevated in white gastrocnemius within 5 h after a single oral dose of metformin in healthy Wistar rats. The apparent temporal sensitivity of AMPK activity to metformin treatment coupled with the powerful anti-diabetic action of metformin suggests that a perhaps a single dose of metformin may elicit similar anti-diabetic effects as chronic treatment. However, there are no published studies to date that have investigated the effect of a single dose of metformin on whole body glucose tolerance in either animal models or humans. Furthermore, it appears that the metabolic action of metformin can occur independently of AMPK in the myocardium, indicating that perhaps AMPK is not an essential contributor to the anti-diabetic action of metformin (61).

CHAPTER 2: PURPOSE AND HYPOTHESES

Purpose

The purpose of this study was to determine if a single oral dose of the anti-diabetic drug metformin can improve whole body glucose tolerance in the male fatty, insulin resistant Zucker rat model concomitant with altered mitochondrial oxidant production.

Hypotheses:

In Zucker fa/fa rats, a single oral dose of metformin will:

1) Increase whole body glucose clearance in response to an oral glucose tolerance test (OGTT).

2) Decrease mitochondrial H_2O_2 emission during succinate and palmitoyl-carnitine supported respiration.

3) Not alter mitochondrial oxygen consumption during respiration supported by pyruvate/malate or palmitoyl-carnitine/malate.

4) Improve insulin signaling.

5) Not alter AMPK signaling.

CHAPTER 3: METHODS

Animals

The male fatty (fa/fa) Zucker rats were obtained based on the severe obesity and insulin resistance that occurs in these animals between 8-12 weeks of age. The fatty Zucker rat was chosen because recent work in this laboratory by Kane et al. (39) has demonstrated that 4 weeks of metformin treatment partially restores insulin sensitivity in Zucker fa/fa rats. Therefore, the same animal model of insulin resistance was used to determine the effects of a single dose of metformin on whole body glucose clearance, skeletal muscle mitochondrial energetics, and skeletal muscle insulin signaling.

Treatment Protocol

This study was conducted in accordance with guidelines set forth by the East Carolina University Institutional Animal Care and Use Committee. Twenty-four Zucker fa/fa rats were obtained and housed in a light (12 light: 12 dark) and temperature controlled (37°C) environment and allowed to eat and drink freely.

Animals were fasted overnight (8-10 hours) and then given either an oral dose (320 mg/kg body weight) of metformin (0.5 M dissolved in ddH_2O) or a water gavage (2 ml/kg body weight) between 5-9 am. The volume of water gavage given was equivalent to the volume of solution given to metformin treated animals. Three hours after the "treatment" gavage, animals were given an OGTT (2 g/kg body wt.). After the OGTT, animals were placed back into their respective cages and were allowed to eat and drink freely for >7 days. Pharmacokinetic evidence indicates that metformin is completely removed from the circulation within 12 hours (62).

After the week of washout, animals were again fasted overnight and then given either an oral dose of metformin or a water gavage in the same concentrations as the previous protocol.

However, 2 hours after the "treatment" gavage, each group (water or metformin) was divided into two subgroups (glucose or water) resulting in four groups: water/water, water/glucose, metformin/water, and metformin/glucose. As such, two groups (water/glucose and metformin/glucose) received an oral glucose gavage (2 g/kg body weight) and the other two groups (water/water and metformin/water) received an equivalent volume of water. One hour after the second gavage, the animals were given an intraperitoneal injection of ketamine/xylazine (50 mg/kg bodyweight) to induce deep anesthesia. After anesthesia was confirmed using the toe pinch technique, the red and white gastrocnemius muscles were rapidly removed from the animal and either used for functional mitochondria measurements or flash frozen in liquid nitrogen for future analyses.

Oral Glucose Tolerance Tests

OGTTs were performed 3 hours after a single oral dose of metformin (320 mg/kg body weight) following an overnight fast using an oral glucose gavage (2 g/kg bodyweight). Approximately 1 ml of blood was collected from the lateral tail vein at baseline (minute 0), 15, 30, 60, and 120 minutes after the glucose gavage was administered. Blood glucose was measured using a whole blood glucometer (OneTouch Ultra). For insulin, whole blood was collected in 1.5 ml Eppendorf tubes on ice, centrifuged at 10,000 rpm for 10 minutes, and the serum drawn off and frozen at -20°C. Serum insulin was measured using a rat/mouse ELISA kit (Linco Research). Homeostatic model assessment (HOMA) was calculated with the following formula: HOMA = fasting insulin (μU/ml) x fasting glucose (mM)/22.5.

Preparation of permeabilized muscle fiber bundles

This technique is partially adapted and has been described previously (3). First, the red gastrocnemius muscle was dissected from the animal, and the muscle was separated into bundles

using a dissection microscope (Leica, Bannockburn, IL). Fiber bundles were immersed in buffer X containing (mM): 60 K-MES, 35 KCl, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 Imidazole, 0.5 DTT, 20 Taurine, 5.7 ATP, 15 PCr, 6.56 $MgCl_2$, - 6 H_2O (pH 7.1, 290 mOsm), and separated using micro dissection instruments to maximize surface area. The separated fiber bundles were then incubated in saponin (50 μg/ml) and buffer X for 30 minutes at 4°C to selectively permeabilize the plasma membrane. After permeabilization, the de-energized fiber bundles (PmFBs) were washed for at least 15 minutes in Buffer Z containing: (mM) 110 K-MES, 35 KCl, 1 EGTA, 5 K_2HPO_4, $3MgCl_2$ – 6 H_2O, 5 mg/ml BSA, 0.05 pyruvate and 0.02 malate (pH 7.4, 290 mOsm) on a rotator at 4° C and then stored at 4°C until analysis (<60 minutes).

Mitochondrial O_2 consumption

Oxygen consumption measurements were performed at 37°C in Buffer Z using the Oroboros O2K Oxygraph (Innsbruck, Austria). Each chamber contained 2 ml of Buffer Z with 20 mM creatine monohydrate (to saturate endogenous creatine kinase), 5 mg/mL BSA, and 50 μM blebistatin (Bleb). Bleb has been shown to be a myosin II inhibitor (1), and has been previously demonstrated to improve stability during O_2 consumption experiments in permeabilized muscle fibers (Perry et al., unpublished observations). After the fiber bundles were added, the chambers were sealed and upon signal stabilization, two different protocols were performed for each fiber type (red and white gastrocnemius) to analyze O_2 kinetics.

Protocol 1: An ADP titration with pyruvate/malate as substrates was performed to determine whether metformin inhibits the sensitivity of the system to ADP (K_m) or the maximal (V_{max}) complex I supported respiration. The protocol was performed as follows: i) 5 mM Malate and 2 mM Pyruvate; ii) 25 μM ADP; iii) 100 μM ADP; iv) 250 μM ADP; v) 1 mM ADP; vi) 4 mM ADP. During this protocol, reducing equivalents are derived almost exclusively from NADH

because the lack of succinate in the protocol prevents substantial flux through succinate dehydrogenase (SDH) which will thus limit the production of $FADH_2$. As a result, only forward electron flow through Complex I should occur. *Protocol 2*: Maximal state 3 (ADP stimulated) respiration with Palmitoyl-carnitine/malate (PCM) was performed to determine if a single oral dose of metformin alters fatty acid supported respiration. The protocol was performed as follows: i) 25 μM palmitoyl carnitine + 1 mM malate (PCM_4); ii) 25 μM ADP; iii) 100 μM ADP; iv) 250 μM ADP; v) 1 mM ADP; vi) 4 mM ADP. The first addition (PCM) initiates a state 4 basal, non-phosphorylating respiration (PCM_4). Reducing equivalents are derived from β-oxidation and the TCA cycle. ADP is then added to initiate state 3, phosphorylating respiration (PCM_3).

Methodological Considerations

Under experimental conditions, there are a number of limiting factors to respiration, such as [O_2], substrate flux into the mitochondria, phosphorylation, and bulk electron flow. In the current project, [O_2] in the chamber was controlled for by maintaining [O_2] within a given range (150-220 μM) at all times during the experiment. This is done to avoid a situation where O_2 delivery is limited or in excess which could interfere with respiration independent of the ETS. Substrate flux into the mitochondria was controlled for by providing a maximal saturating concentration of each substrate or by performing a substrate titration during the experiment, effectively determining maximal substrate oxidation in a given protocol. Limitations in ADP transport or phosphorylation occur at either the adenine nucleotide translocase or at the ATP synthase, respectively. Although [O_2] and substrate flux are controlled within the experimental design, the limitations regarding ADP transport and phosphorylation are analyzed as a whole primarily by calculating UCR. A limitation in any of these systems will inhibit respiration relative to maximal uncoupled respiration. Ultimately, assuming maximal substrate oxidation

and ideal [O_2], bulk electron flow is the only limiting factor during maximal uncoupled respiration, and is recognized as the maximal rate of respiration in the given experimental protocols. The protocols address each of the limitations in a step-wise fashion, providing a comprehensive analysis of mitochondrial respiratory function.

At the conclusion of each experiment, PmFBs were freeze-dried in a lyophilizer (Labconco) overnight. After freeze-drying, the fibers were weighed and O_2 consumption was expressed as pmol·sec^{-1}·mg dry weight^{-1}.

Mitochondrial H_2O_2 Emission

Mitochondrial H_2O_2 emission (mH_2O_2) was measured by continuously monitoring oxidation of Amplex Red using a Spex Fluoromax 3 (Jobin Yvon, Ltd.) spectrofluorometer. Chemically, this experiment is based on the oxidation of Amplex Red (Invitrogen) by H_2O_2 in the presence of horseradish peroxidase (HRP) to form resorufin, which fluoresces at a 1:1 stoichiometry with [H_2O_2], providing a direct measure of H_2O_2 emission. The mH_2O_2 assay was set up by adding 300 µl of Amplex Red reagent with 1 µl HRP in a quartz cuvette. Two protocols were employed for each fiber type (red and white gastrocnemius).

Protocol 1: The first protocol performed consisted of the initial addition of 3 mM succinate followed by an ADP titration. In order, the following substrates were added during the experiment: i) 3 mM Succinate; ii) 25 µM ADP; iii) 100 µM ADP; iv) 250 µM ADP; v) 500 µM ADP; vi) 1 mM ADP. This protocol was used to induce a high rate of reverse electron flow through Complex I, and then to test the sensitivity of the mitochondria to [ADP] in a way similar to what was done while measuring O_2 kinetics. Although ADP was added for protocols measuring either O_2 consumption or H_2O_2 emission, it is important to note that the addition of [ADP] will decrease membrane potential ($\Delta\Psi$), which will decrease mH_2O_2 concomitant with

greater O_2 consumption. Taken together, mitochondrial sensitivity to [ADP] under both of these conditions was performed to determine if metformin administration altered the dynamic relationship between adenine transport/phosphorylation and mH_2O_2.

Protocol 2: The second protocol was done in two steps, first palmitoyl-carnitine and malate was added to measure fatty acid induced mH_2O_2. The purpose of this part of the protocol was to measure fatty acid supported mH_2O_2 using an "activated" form of palmitate. In order for fatty acids to enter the mitochondria for β-oxidation they must be converted to palmitoyl-carnitine and then transported into the mitochondrial matrix via CPT 1/2. Immediately afterwards, the fiber was removed, washed in fresh Buffer X and then added into a fresh cuvette. At this point, 3 mM succinate was added, followed by rotenone. The purpose of the second step of the protocol was to see if metformin treatment inhibited complex I reverse electron flow to a similar degree as rotenone.

At the conclusion of each mH_2O_2 experiment, the fiber bundles were washed in ddH_2O, freeze-dried and weighed. H2O2 emission data is expressed for each substrate condition as μmol H_2O_2/min/mg dry wt.

Preparation of tissue for muscle protein

A portion of red gastrocnemius collected at dissection was frozen in liquid N_2 and powdered by pulverization. To prepare samples for GSH analysis, 100mg of tissue was added to a test tube containing 1 ml of homogenization buffer consisting of (mM): 10 Tris, 1 EDTA, 1 EGTA, 2 BaO, 2 Sodium Pyrophosphate (NaPPi), 5 sodium fluoride (NaF), and protease inhibitor cocktail at pH 7.2. Tissue was then homogenized using a Polytron SC-250 set at 25,000 rpm. After homogenization, 1% Triton X-100 was added to the suspension, vortexed and put on ice for 5 minutes. The tubes were then pelleted by centrifugation at 10,000 rpm for 10

minutes, supernatant decanted and the pellet stored at -80°C until muscle protein analysis is performed.

Glutathione and Glutathione Disulfide analysis

Total GSH (tGSH) and GSSG were measured using reagents and a calibration kit provided (Oxis Research) according to manufacturer's instructions, with small modifications. For measurement of GSSG, samples were homogenized in the presence of 0.3 mM Methyl-2-Vinylpyridinium triflate to scavenge all reduced thiols present in the sample. From tGSH and GSSG, GSH/GSSG was calculated using the following formula:

GSH/GSSG ratio = (tGSH – (2*GSSG))/ GSSG

Analysis of protein signaling

To extract proteins from tissues for the analysis of insulin signaling factors, frozen muscle was pulverized in liquid nitrogen with mortar and pestle, then placed in 10 volumes (~100 mg/1 ml buffer) of homogenization buffer (mM): 50 HEPES, 50 NaPPi, 100 NaF, 10 EDTA, 10 NaO, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma, St. Louis, MO) on ice. After centrifugation for 10 min at 10,000 rpm, supernatants were taken and protein content was determined using a BCA protein assay (Pierce, Rockford, IL). An equal amount of protein was loaded into 12.5% Tris HCl gels, and gel electrophoresis was performed at 150 mV for ~1 hour. The gels were then moved to a transfer apparatus in ice cold running buffer and proteins were transferred to a PVDF membrane for 2 hours at 100mV). Primary antibodies were then applied overnight in the following dilutions: total Akt (1:2000), Akt Ser^{473} (1:2000), total AMPK (1:1000), and AMPK Thr^{172} (1:1000). Total Akt, total AMPK, and AMPK Thr^{172} antibodies were purchased from Cell Signaling (Beverly, MA). Akt Ser^{473}

was purchased from Santa Cruz Biotech (Santa Cruz, CA). Following incubation with primary antibodies, blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody purchased from Cell Signaling. Horseradish peroxidase activity was assessed with Western Lightning ECL solution (Lumiglo and Peroxide, Cell Signaling, San Francisco, CA), and exposed to blue sensitive x-ray film. Exposure times were optimized for each particular experiment. The resulting image was scanned and band densitometry was assessed with Gel Pro Analyzer software (Media Cybernetics, Silver Spring, MD). Protein phosphorylation was calculated as the ratio of phosphorylated/total protein (i.e. pAkt/tAkt), normalized to the control fasted (water/water) animals and reported as % change from control.

Statistical analysis

Data is presented as mean $\pm$ SEM. Data was analyzed using two-tailed unpaired t-test for OGTT data and either one-way or two-way ANOVAs for all other measurements. The significance level was set at $p < 0.05$.

CHAPTER 4: RESULTS

A single oral dose of Metformin improves Whole Body Glucose Tolerance.

Glucose tolerance tests were performed on Zucker fa/fa rats 3 hours after an oral dose of metformin. Fasting HOMA was significantly lower in metformin treated rats compared to control (Figure 1A - Control 3.96 $\pm$ 0.44 vs. Metformin 2.75 $\pm$ 0.28; $p = 0.03$). This difference appears to be attributed to significantly lower fasting glucose in metformin treated animals (Figure 1B - Control - 121 $\pm$ 4 vs. Metformin - 97 $\pm$ 4; two-tailed t-test $p \leq 0.0005$) whereas no difference in fasting insulin (Figure 1C - Control - 13.03 $\pm$ 1.28 vs. Metformin - 11.36 $\pm$ 0.97; two-tailed t-test $p = 0.31$) was observed. Glucose AUC was significantly lower (35%) in metformin treated rats compared to the control group (Figure 2A - Control 33310 vs. Metformin 21590; $p \leq 0.0001$) whereas insulin AUC was not significantly different between groups (Figure 2B). Taken together, these data provide evidence that a single dose of metformin improves whole body glucose tolerance, similar to the improvements in glucose tolerance observed with metformin treatment performed for several weeks (39).

Mitochondrial O_2 Consumption is not impaired by acute Metformin treatment

Mitochondrial oxygen consumption was measured in both red and white gastrocnemius muscle of Zucker fa/fa rats three hours after an oral dose of metformin or water and then one hour after a glucose gavage or a second water gavage. Mitochondrial oxygen consumption was measured during either pyruvate/malate (PM) or palmitoylcarnitine/malate (PCM) supported respiration utilizing an ADP titration to derive K_m and V_{max}. As hypothesized, there were no statistically significant differences in K_m or V_{max} between any groups during PM or PCM supported respiration in red or white gastrocnemius. Interestingly, although the theoretical V_{max} for PM supported respiration was not different between water/water and metformin/water in red

gastrocnemius, the maximal ADP (4 mM) stimulated rate of respiration was significantly different between water/water and metformin/water (water/water – 708.3 $\pm$ 95.70 vs. metformin/water – 1054.1 $\pm$ 271.51; $p < 0.05$).

Cellular Redox State is altered by a single oral dose of Metformin

The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was measured and used as an index of cellular redox state. White gastrocnemius GSH/GSSG was significantly decreased an hour after a glucose gavage in control animals, but in contrast, GSH/GSSG was significantly elevated in metformin treated animals in response to glucose (Figure 3A). However, contrary to expectations, water/water animals displayed a significantly greater GSH/GSSG ratio than metformin/water animals, suggesting that perhaps metformin has a deleterious effect on cellular redox state during fasting. The altered GSH/GSSG ratios observed in the white gastrocnemius were not associated with significant differences in GSSG, which indicates that this response may not be exclusively due to alterations in mitochondrial oxidant production *per se*. Furthermore, although significant differences were observed in the GSH/GSSG ratio in white gastrocnemius, no differences were observed in total GSH, GSSG, or GSH/GSSG in red gastrocnemius.

Mitochondrial H_2O_2 emission is not decreased in skeletal muscle by a single oral dose of Metformin

Mitochondrial H_2O_2 emission (mH_2O_2) was measured in both red and white gastrocnemius of Zucker fa/fa rats induced by either succinate or palmitoylcarnitine. No significant differences between groups were observed in red gastrocnemius during succinate or PCM induced mH_2O_2 (Figure 3B). Additionally, no significant differences between groups were observed in white gastrocnemius during succinate or PCM induced mH_2O_2. After the addition of succinate, an

ADP titration was performed to determine if metformin altered the reduction of mH_2O_2 in response to [ADP] in red gastrocnemius. The percent (%) change was calculated by subtracting mH_2O_2 at a given [ADP] by the maximal succinate induced mH_2O_2 rate, then dividing by the maximal induced mH_2O_2 rate. No differences were observed between groups in mH_2O_2 sensitivity of red gastrocnemius to [ADP].

Akt but not AMPK phosphorylation is altered by acute Metformin treatment

To determine the effects of a single dose of metformin on insulin signaling, phosphorylated (Ser^{473}) and total Akt were measured by immunoblotting using equal amounts of protein in red and white gastrocnemius. As expected, glucose treatment resulted in significantly elevated Akt Ser^{473} phosphorylation in red and white gastrocnemius compared to controls (181% and 280%, respectively), indicating activation of insulin signaling (Figure 4A). Interestingly, metformin treatment resulted in elevated Akt Ser^{473} phosphorylation independent of glucose in both red and white gastrocnemius (323% and 176%, respectively) compared to fasted controls. Although both glucose and metformin increased Ser^{473} phosphorylation of Akt alone, treatment with both glucose and metformin did not have an additive effect.

The current dogma with respect to metformin indicates that an increase in AMPK phosphorylation and/or activity is integral to its' anti-diabetic effects (54, 81). However, recent evidence indicates that activation of AMPK may not be required for particular metabolic effects of metformin (61, 72). Therefore, to determine if a single dose of metformin causes increased AMPK phosphorylation, phosphorylated (Thr^{172}) and total AMPK were measured by immunoblotting in red and white gastrocnemius. No significant differences were observed between any groups in AMPK phosphorylation in response to glucose or metformin in red or white gastrocnemius (Figure 4B). This data indicates that a single oral dose of metformin does

not cause an increase in AMPK phosphorylation, and furthermore that the anti-diabetic effects of metformin may not rely on AMPK, at least in skeletal muscle.

CHAPTER 5: DISCUSSION

Summary of findings

The results of the current study demonstrate a dramatic improvement in whole body glucose tolerance three hours after a single oral dose of metformin in Zucker *fa/fa* rats which is independent of changes in mH_2O_2 emitting potential or AMPK phosphorylation in red and white gastrocnemius. Cellular redox state was differentially altered in response to glucose and/or metformin in white gastrocnemius, but no changes were observed in red gastrocnemius. Interestingly, the same trend seen in white gastrocnemius GSH/GSSG was observed in the mH_2O_2 emitting potential, although the latter was not statistically significant. The most significant finding of the current study is that a single dose of metformin dramatically improves whole body glucose tolerance in Zucker fa/fa rats, but does so independent of altered mitochondrial respiration, mitochondrial oxidant production, or AMPK phosphorylation. Further investigation is needed to elucidate the mechanism responsible for the anti-diabetic action of metformin in skeletal muscle.

Improved Glucose Tolerance after a single dose of Metformin

Fasting glucose is primarily regulated by hepatic glucose output, and thus the 20% improvement in fasting glucose observed after a single oral dose of metformin in this study fits with previous data which has shown a dramatic reduction in hepatic glucose output in response to chronic metformin treatment in humans (37). The improvement in whole body glucose tolerance observed in this study was not as great as the ~60% decrease in glucose AUC observed after 4 weeks of metformin treatment in Zucker fa/fa rats by Kane et al. (39). However, it appears that a single dose of metformin resulted in a clinically relevant improvement in whole body glucose tolerance in both the fasted (20%) and fed (35%) states. Furthermore, consistent

with previous findings from our lab, metformin treatment did not alter fasting serum insulin or total insulin area under the curve during an OGTT. These data taken together are strong evidence to demonstrate the anti-diabetic action of metformin in skeletal muscle, which may require insulin (37), but can alter glucose tolerance independent of changes in insulin secretion as seen with chronic metformin treatment (39).

Metformin acts independently of alterations in Mitochondrial Respiration to improve Whole Body Glucose Tolerance

Previous research has suggested that the anti-diabetic effects of metformin may be due to inhibition of Complex I supported respiration (20, 55), however, many of these studies used metformin at concentrations many times higher than those which exist *in vivo*. In the current study, a single dose of metformin did not alter maximal state 3 mitochondrial respiration in red or white gastrocnemius using pyruvate/malate or palmitoylcarnitine/malate as substrates. This data suggests that skeletal muscle mitochondrial respiration is not altered after a single dose of metformin treatment, and furthermore that it is not required for the anti-diabetic effects of metformin treatment. In further support of these findings, Kane et al. (39) recently demonstrated that the concentration of metformin needed to inhibit electron flow in the forward direction (respiration) was ~100 times greater than the concentration needed to inhibit reverse electron flow (oxidant generation). Taken together, it appears fairly evident that although metformin is capable of inhibiting mitochondrial respiration *in vitro* at high concentrations, it is unlikely that metformin treatment alters skeletal muscle mitochondrial respiration *in vivo*.

A single dose of Metformin alters Cellular Redox state in a Skeletal Muscle Fiber Type specific manner

Recent evidence has indicated that a potential mechanism for metformin to elicit anti-diabetic effects is through the inhibition of mitochondrial oxidant production, particularly reverse electron flow through Complex I (9, 55). In the current study, we tested whether a single dose of metformin altered mitochondrial H_2O_2 production induced by palmitoylcarnitine/malate or succinate in red and white gastrocnemius. Contradictory to the hypotheses set forth, a single dose of metformin did not appear to significantly alter mitochondrial oxidant production in red gastrocnemius.

Perhaps the most interesting finding of the current study was observed in white gastrocnemius cellular redox state. As expected, water/glucose animals displayed a more oxidized GSH/GSSG ratio in white gastrocnemius compared to water/water, indicating that the cell redox environment became more oxidized in response to glucose. However, when compared to the control fasted animals, metformin/water animals had a more oxidized cellular redox state (decreased GSH/GSSG) in white gastrocnemius. However, when metformin and glucose treatments were given together (metformin/glucose group), the cellular redox environment was significantly more reduced (higher GSH/GSSG) compared to metformin/water animals, indicating a protective effect of metformin. Although these results seem contradictory, there is evidence from previous studies to explain how/why this phenomenon may occur. As mentioned previously, a key component of the metabolic action of metformin is thought to be through an increase in AMPK phosphorylation or activity, although in the current study, phosphorylation of AMPK did not appear to be elevated. One observed result of metformin treatment (presumed to be AMPK dependent) is an increase in fatty acid oxidation and transport, which occurs through

phosphorylation of ACC, resulting in a decrease in malonyl-CoA levels (15). Malonyl CoA is a competitive inhibitor of carnitine palmitoyl transferase 1 (CPT-1), and thus decreased levels of malonyl CoA result in greater fatty acid transport via CPT-1 (60). Therefore, although perhaps not through an AMPK-dependent mechanism, if fatty acid transport and/or oxidation was elevated in the fasted state by metformin treatment, it could result in a more oxidized cellular redox environment due to elevated fatty acid supported mitochondrial oxidant generation (3). The dichotomy between red and white gastrocnemius may be explained by the different energetic properties of these two tissues, with red gastrocnemius possessing far greater mitochondrial density than white gastrocnemius. Therefore, perhaps the same concentration of glucose (or metformin) affects these two tissues differently, which makes the response to metformin treatment difficult to compare between different fiber types.

A single dose of Metformin can mimic Insulin signaling in the Fasted state

The canonical insulin signaling cascade in skeletal muscle consists of a series of phosphorylation events beginning at the insulin receptor and culminating with GLUT4 translocation to the plasma membrane. At the epicenter of a variety of different signaling cascades, including insulin signaling, is the pro-survival kinase Akt. In the current study, Akt phosphorylation was measured as an index of insulin signaling in red and white gastrocnemius. Consistent with previous findings, glucose treatment increased Akt phosphorylation, indicating an activation of insulin signaling. Interestingly, metformin treatment alone significantly increased Akt phosphorylation in both red and white gastrocnemius, yet combined glucose and metformin did not have an additive effect on Akt phosphorylation on either tissue. This data is provocative because previous findings indicate that insulin is necessary for metformin induced elevations of whole body glucose clearance (77). However, based on the current findings, it

appears that perhaps insulin is not required for the anti-diabetic action of metformin *in vivo*, although this is based solely on protein signaling data since no direct measurements of skeletal muscle glucose transport were performed in the current study.

Limitations

Two types of limitations can be identified in this study: limitations based on the experimental models used and specific methodological limitations based on particular protocols. The primary limitations regarding experimental model and design are: lack of skeletal muscle specific glucose transport data and the use of a genetically predisposed obese animal as opposed to a naturally occurring type 2 diabetic animal. The effects of a single dose of metformin on glucose tolerance in human or animal has only been assessed in one study to our knowledge (57). Perriello et al. (57) found that treating humans with a single dose of metformin resulted in dramatic reductions in hepatic glucose output with no alterations in peripheral glucose uptake. Ultimately, the current study provides inferential evidence that peripheral glucose transport was altered by a single dose of metformin based on the glucose AUC data, but when considering the previously reported contribution of hepatic glucose output in response to metformin treatment, specific skeletal muscle glucose transport data is needed. The second major design limitation of the current study was the use of the Zucker fa/fa rat instead of a high fat fed rat. The Zucker fa/fa rat is a leptin receptor deficient rat, meaning that leptin is released but cannot bind to receptors and act accordingly on feeding behavior, resulting in a hyperphagic animal that becomes insulin resistant between 10-12 weeks of age. Previous research has demonstrated that metformin treatment improves leptin sensitivity in leptin-resistant high fat fed animals (42), and therefore the use of a leptin receptor deficient animal model lacks some strength in translating the animal data obtained in the current study to future humans studies. The third and final

limitation related to design was the low group sizes as a result of unforeseen circumstances. This resulted in group sizes of 3-4 in our functional mitochondria experiments, and yet another unfortunate event weakened the usefulness of stored frozen tissue, resulting in group sizes of 2-3 for the western blot data presented in this study. Therefore, all data except for the OGTT (glucose and insulin) and GSH/GSSG in this paper should be considered pilot data.

Specific methodological limitations over the course of a study are commonplace as the development of new information and techniques become available. The methodological limitations of the current study are primarily regarding the measurement of mitochondrial oxidant generation. Specifically, based on the experimental conditions used, it is possible that the relative contribution of sites other than Complex I to altered mitochondrial oxidant production in response to metformin treatment may have been underestimated. Previous research has indicated that Complex I was the primary target of metformin treatment (9). However, recent evidence from Seifert et al. (66) indicates that superoxide can be produced from the electron transferring flavoprotein as well as Complex III during fatty acid oxidation, and is therefore relevant to this study. Furthermore, inclusion of exogenous CuZnSOD effectively doubles fatty acid induced H_2O_2 emission in isolated mitochondria (66). This issue is paramount because our laboratory has used exogenous copper-zinc superoxide dismutase (CuZnSOD) in previous studies, the primary cytosolic superoxide scavenger *in vivo*, during the measurement of H2O2 emission, but exogenous CuZnSOD was not used in the current study because the contribution of Complex III to H2O2 emission was thought to be minimal (4). This means that the relative contribution of alternate site oxidant generation may have been underestimated in the current study, and is a potential explanation for many of the discrepancies found between previous studies in our laboratory and the current study. Therefore, although the results of the

current study did not fit some of the proposed hypotheses, methodological insight has been gained from this project and further investigation needs to be done to determine the topology of oxidant generation in response to glucose and/or metformin treatment.

Future Directions

There are abundant opportunities in the future to study metformin, particularly because it is the most widely prescribed drug to type 2 diabetics while the mechanism of action is still largely unknown. The current study has revealed novel descriptive responses to a single oral dose of metformin in the Zucker fa/fa rat, particularly with respect to whole body glucose tolerance and alterations in cellular redox state by metformin treatment. Potential future projects based on the insight gained from the current study should include direct measurements of hepatic glucose output and skeletal muscle glucose transport using radiolabeled tracers, and use either humans or high fat fed animal models instead of genetically obese animal models. Investigating the role of AMPK as a biological target of metformin treatment in insulin sensitive tissues remains viable to study, as well as further exploring the insulin independent activation of insulin signaling proteins such as Akt, and how metformin treatment could affect protein synthesis through differential regulation of AMPK and Akt.

Conclusions

The current study tested the hypothesis that a single oral dose of metformin in genetically hyperphagic Zucker fa/fa rats would improve whole body glucose tolerance and be associated with decreased mitochondrial oxidant generation and improved cellular redox state. Based on the results of the study, it can be concluded that a single dose of metformin results in improvements in whole body glucose tolerance that may be independent of changes in cellular

redox state or mitochondrial oxidant generation in red gastrocnemius, but related to altered cellular redox state in white gastrocnemius. Metformin treatment in the fasted state may be detrimental to cellular redox state in white gastrocnemius, but should be tested further to determine if this is physiologically relevant. Although threonine phosphorylation of AMPK was not increased in red or white gastrocnemius, serine phosphorylation of Akt was increased, indicating that metformin can mimic insulin to a certain extent in vivo and does not appear to rely on AMPK. Cumulatively, the insight provided by the current study is descriptive and provides a solid foundation on which to pursue future studies regarding the acute metabolic alterations that occur in response to metformin treatment *in vivo*.

Figure 1: EFFECTS OF A SINGLE ORAL DOSE OF METFORMIN ON FASTING GLUCOSE AND INSULIN IN ZUCKER fa/fa RATS

HOMA was calculated (A) (n=11/group) from fasting blood glucose (B) (n=12/group) and serum insulin (C) (n=11/group) which were measured after an overnight fast in Zucker fa/fa rats after either an oral dose of metformin (320 mg/kg body wt.) or an equivalent volume of water. The results demonstrate the potential for metformin treatment (black bars) to improve HOMA (A - * $P \leq 0.05$) and decrease fasting glucose (B - *** $P \leq 0.0001$) compared to controls (white bars). Data is represented as the mean $\pm$ SEM.

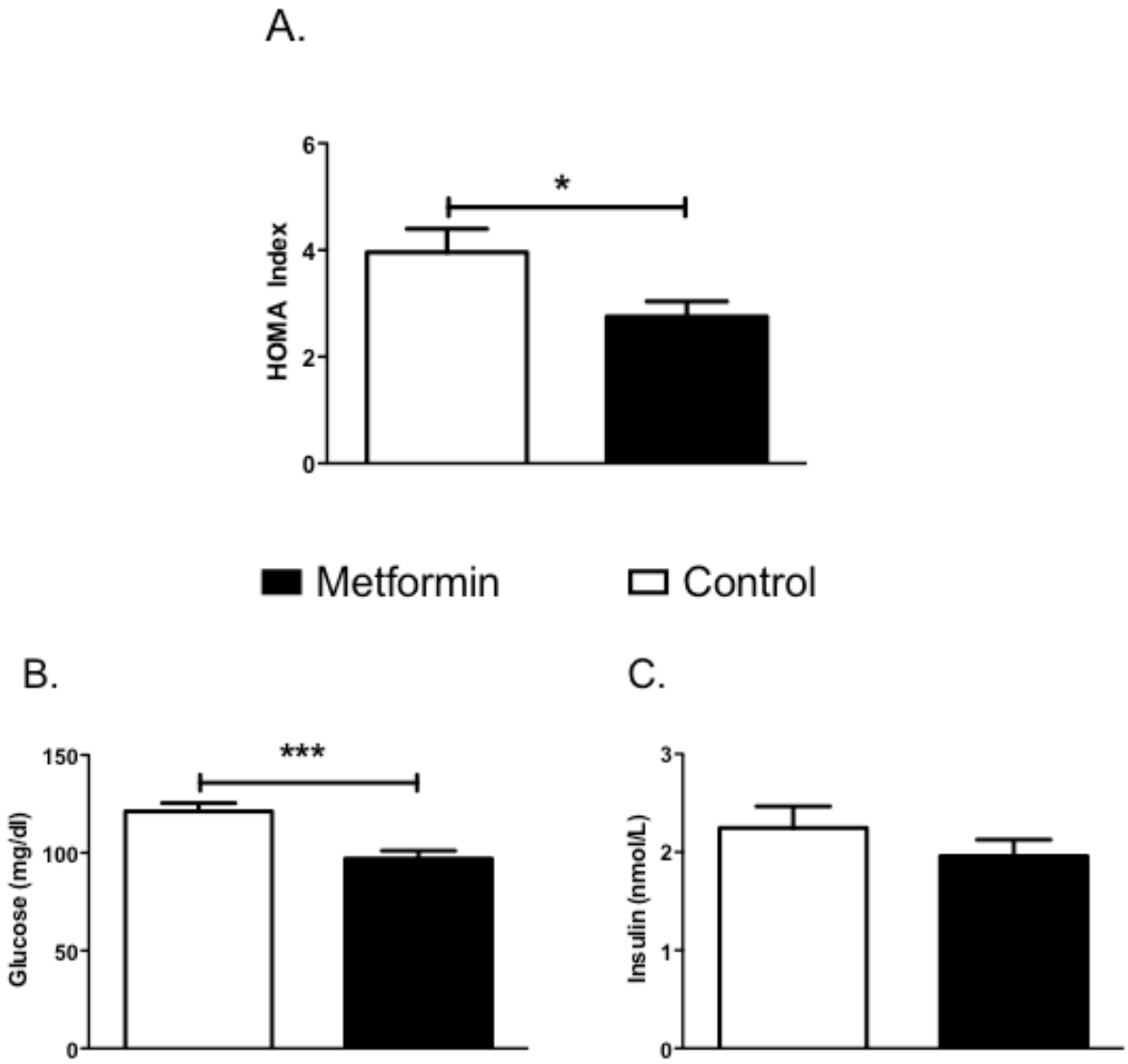
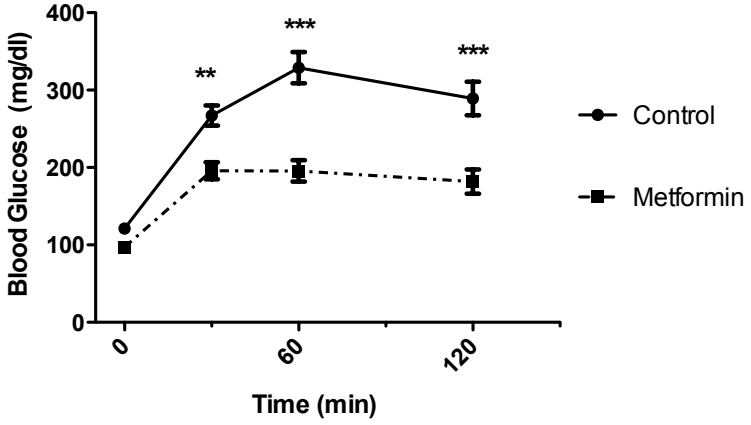

A.
HOMA Index
6
4
2
0
*
Metformin
Control
B.
Glucose (mg/dl)
150
100
50
0
***
C.
Insulin (nmol/L)
3
2
1
0

Figure 2: EFFECTS OF A SINGLE DOSE OF METFORMIN ON WHOLE BODY GLUCOSE TOLERANCE IN OBESE ZUCKER fa/fa RATS

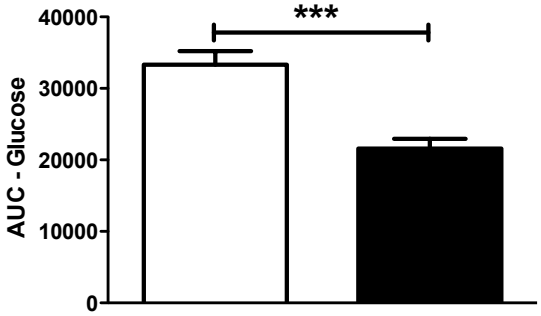
Time course of blood glucose (A) during an OGTT (n=12/group). Results indicate that a single dose of metformin decreases blood glucose at every time point beyond basal when assessed with 2-way ANOVA and Bonferroni post-hoc test (** $P < 0.01$, *** $P < 0.001$). Glucose (B) (n=12/group) and insulin (C) (n=11/group) total area under the curve (AUC) from an oral glucose tolerance test (2g/kg bodyweight) 3 hours after a single oral dose of metformin (320mg/kg bodyweight). Results demonstrate that metformin treatment (black bars) improves whole body insulin sensitivity compared to controls (white bars) evidenced by decreased glucose AUC (*** $P < 0.0001$), but no significant difference in insulin AUC. Data is represented as the mean $\pm$ SEM.

A.


400
300
200
100
0
Blood Glucose (mg/dl)
**
***
***
0
60
120
Time (min)
Control
Metformin


Control
Metformin

B.


40000
30000
20000
10000
0
AUC - Glucose
***

C.

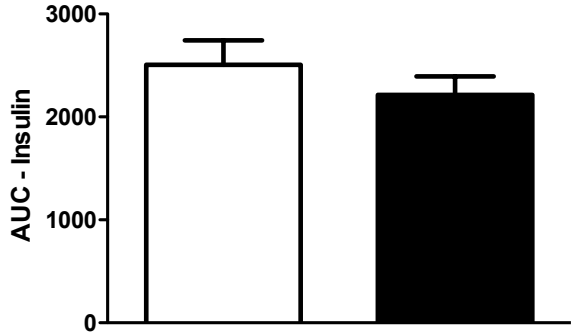
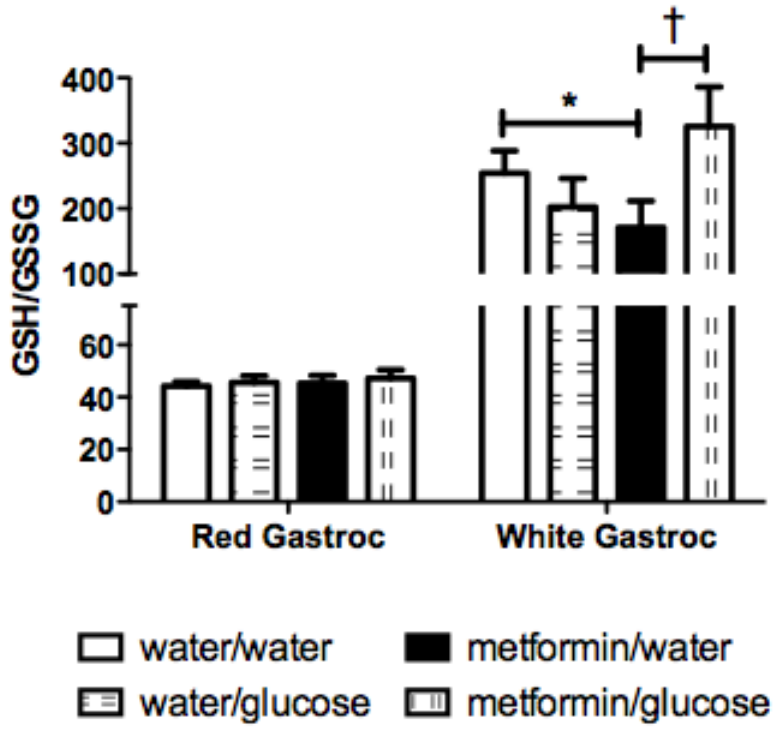

3000
2000
1000
0
AUC - Insulin

FIGURE 3: EFFECTS OF A SINGLE DOSE OF METFORMIN ON CELLULAR REDOX STATE AND MITOCHONDRIAL OXIDANT PRODUCTION IN RED AND WHITE GASTROCNEMIUS OF ZUCKER fa/fa RATS

Cellular redox state (A) (n=6/group) and mitochondrial oxidant production (B) (n=3-4/group) were measured under four conditions (see *Methods*). The first gavage was performed 3 hours prior to sacrifice and consisted of either metformin (320 mg/kg body wt.) or an equivalent volume of water, the second gavage was performed 1 hour before sacrifice and consisted of either glucose (2 g/kg body wt.) or an equivalent volume of water. Therefore, four groups were studied (gavage 1/gavage 2): water/water (white bars), water/glucose (horizontal line bars), metformin/water (black bars), and metformin/glucose (vertical line bars). The results indicate that red gastrocnemius cellular redox state is not altered by glucose or metformin treatment. However, in white gastrocnemius, metformin treatment alone resulted in a more oxidized cellular redox environment (* $P \leq 0.05$ vs. water/water, † $P \leq 0.05$ vs. metformin/water), but when combined with glucose, metformin does not appear to alter cellular redox state. Succinate supported mH2O2 was not significantly altered by metformin or glucose treatment in red or white gastrocnemius. Together, this data indicates that there may be a fiber type specific amplitude of alterations in cellular redox state in response to metformin treatment. Data are represented as the mean $\pm$ SEM. (B is considered pilot data)

A.


GSH/GSSG
400
300
200
100
60
40
20
0
*
†
Red Gastroc
White Gastroc
water/water
metformin/water
water/glucose
metformin/glucose

B.

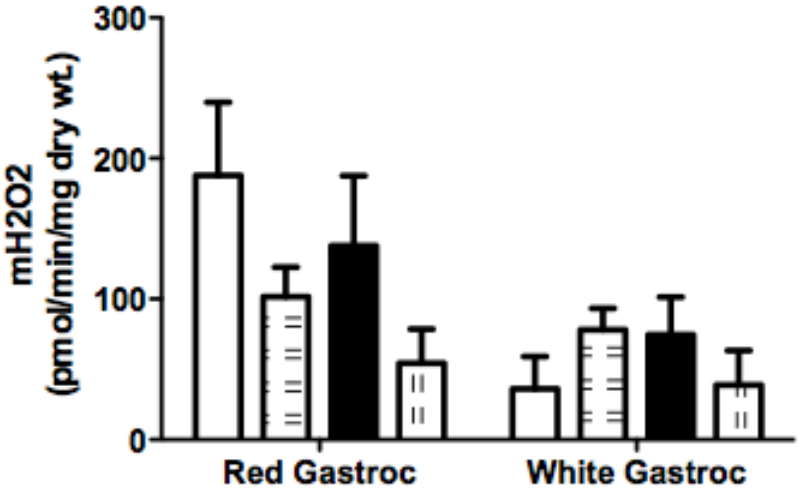
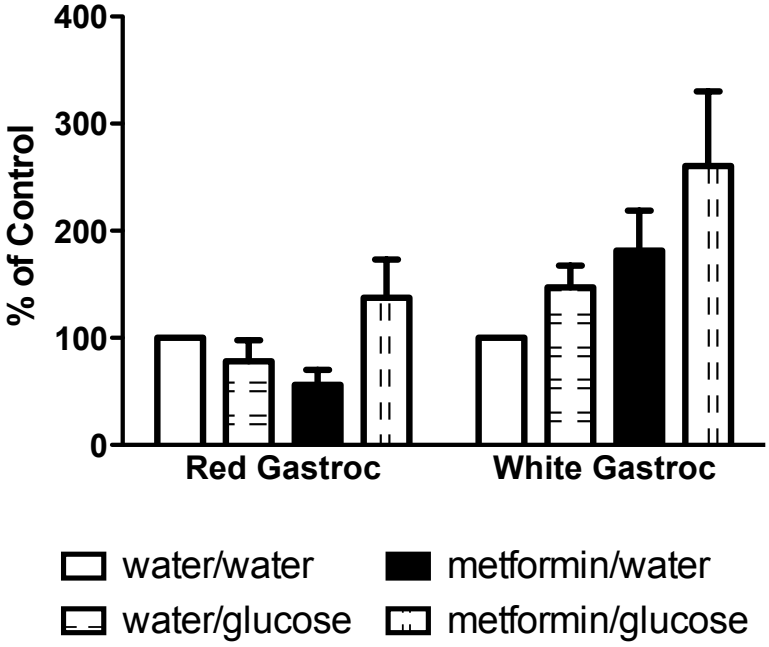

mH2O2
(pmol/min/mg dry wt.)
300
200
100
0
Red Gastroc
White Gastroc

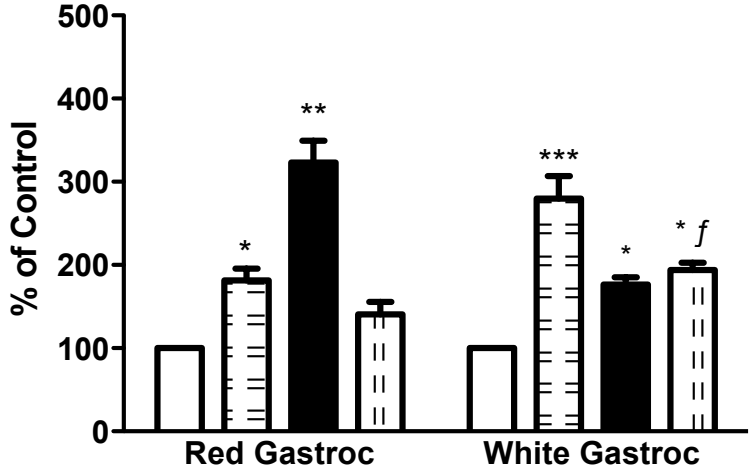
FIGURE 4: EFFECTS OF A SINGLE ORAL DOSE OF METFORMIN ON PROTEIN SIGNALING IN RED AND WHITE GASTROCNEMIUS OF ZUCKER fa/fa RATS

To assess if a single dose of metformin altered protein signaling of Akt (A) and AMPK (B), phosphorylated and total content of these proteins was measured by immunoblotting (see *Methods*) and reported as phosphorylated/total (A and B – n=3/group). Four groups were studied (gavage 1/gavage 2): water/water (white bars), water/glucose (horizontal line bars), metformin/water (black bars), and metformin/glucose (vertical line bars). The results indicate that glucose or metformin treatment alone are both capable of increasing Akt Ser^{473} phosphorylation in both red (* $P \leq 0.05$ vs. water/water, ** $P \leq 0.01$ vs. water/water) and white gastrocnemius (* $P \leq 0.05$ vs. water/water, *** $P \leq 0.001$ vs. water/water, *ƒ* $P \leq 0.05$ vs. water/glucose), but that glucose and metformin treatment together does not result in additive Akt phosphorylation in either tissue. Conversely, glucose or metformin treatment alone did not result in an increase in AMPK phosphorylation, nor did combining the treatments. This suggests that metformin can mimic insulin stimulation at the level of Akt, and that perhaps the metabolic action of metformin may occur independently of AMPK. (A and B are considered pilot data)

A. **pAMPK / tAMPK**



B. **pAkt / tAkt**



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APPENDIX A: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE ANIMAL USE PROTOCOL APPROVAL FORM

East Carolina University.

1807-2007 CENTENNIAL

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 4, 2009

Darrell Neufer, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Neufer:

Your Animal Use Protocol entitled, "Mitochondrial Bioenergetics and Metabolic Disease - Rats," (AUP #Q238a) was reviewed by this institution's Animal Care and Use Committee on December 3, 2009. The following action was taken by the Committee:

"Require the following modification to receive approval"

1. Please reconcile rat treadmill familiarization time. Page 7 indicates 5 minutes while page 15 indicates 10 minutes.

This AUP approves total animal numbers, strains of animals, and procedures that can be done in various combinations. **As each specific experiment is designed, please submit an amendment to the IACUC specifying number of animals per experimental group, strain(s), and procedures to be done.**

Please revise the original Animal Use Protocol to address this required modification and return to Ms. Janine Davenport (212 Life Sciences Building).

Please contact me if I can be of further assistance.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure