

The Role of Angiotensin II in Skeletal Muscle Metabolism

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Abstract

Hypertension and diabetes have long been closely linked. As such, the major player in the renin, angiotensin system, angiotensin II, has recently been investigated for its effects on metabolism and diabetes. Since skeletal muscle is one of the most metabolically active tissues, this study investigates the effects of angiotensin II specifically on skeletal muscle. In this study, L6 skeletal muscle cells were treated with angiotensin II for either 3 or 24 hours and a number of effects were investigated. Fatty acid oxidation and lipid synthesis was measured using [1-¹⁴C]-palmitate, glucose oxidation and glycogen synthesis were measured using ¹⁴C-glucose. In addition, mitochondrial oxidative capacity was measured using an XF 24 Flux Analyzer (Seahorse Bioscience) and reactive oxygen species measured using confocal microscopy. The clinical study involving the drug Benicar ® investigated the metabolic effects of blocking angiotensin II on skeletal muscle fatty acid oxidation, glucose oxidation, and oxidative and glycolytic enzyme activity. In L6 cells, angiotensin II significantly reduced fatty acid oxidation after 24 hours ($p < 0.01$) and 3 hours ($p < 0.001$) if angiotensin II was present during oxidation experiments. It also significantly reduced mitochondrial oxidative capacity ($p < 0.05$) after 24 hours and significantly increased reactive oxygen species production ($p < 0.05$) over 3 hours. The clinical study showed no significant effects of Benicar® on fatty acid or glucose oxidation or any enzyme activities.

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List of Definitions

- 1. BMI – Body Mass Index**
- 2. Ang II – Angiotensin II**
- 3. RAS – Renin-Angiotensin System**
- 4. ROS – Reactive Oxygen Species**
- 5. GLUT4 – Glucose Transporter 4**
- 6. IRS-1 – Insulin Receptor Substrate 1**
- 7. ACE – Angiotensin Converting Enzyme**
- 8. ARB – Angiotensin II Receptor Blocker**
- 9. PPAR γ - Peroxisome Proliferator-Activated Receptor Gamma**

Chapter 1: Introduction

Introduction

Obesity, defined as a body mass index (BMI) greater than 30 kg/m^2 , has been on the rise in the United States and currently 33% of American adults are considered obese (Ogden, Yanovski et al. 2007). This is putting increasing strain on the country's health care system as costs continue to rise. Obesity is associated with many comorbidities, including type II diabetes and cardiovascular disease, with the latter being the leading cause of death in the United States. Thus, in an effort to identify novel therapies, considerable research effort has gone into investigating the biochemical pathways involved in these diseases.

Obesity and hypertension are both criterion for diagnosing metabolic syndrome and are very closely linked, but little is known about which biochemical pathways cause these conditions (Eckel, Grundy et al. 2005). Obesity is also associated with metabolic inflexibility, which is characterized by an inability of the skeletal muscle to easily transition between substrates (Kelley and Mandarino 2000; Stump, Henriksen et al. 2006). Normal weight subjects, when challenged with a hyperinsulinemic-euglycemic clamp, are able to increase the utilization and storage of glucose and markedly reduce fatty acid oxidation (Stump, Henriksen et al. 2006). Conversely, in obese and insulin-resistant individuals there is an inability to increase glucose oxidation and storage in response to hyperinsulinemia and fatty acid oxidation remains unchanged. Metabolic inflexibility is also characterized by skeletal muscle and adipose tissue insulin resistance. Recent work has highlighted the potential role of the renin-angiotensin system (RAS), specifically angiotensin II (Ang II), in the development of insulin resistance. Treatment of animals and cells with Ang II has been shown to increase production of reactive

oxygen species (ROS) via activation of NADPH oxidase (Puri, Avantiaggiati et al. 1995; Herrera and Garvin 2010; Phillips, Pechman et al. 2010). ROS has many adverse effects including cellular damage, mitochondrial damage, and a reduction in oxidative capacity, as well as disruption of the insulin signaling cascade (Wei, Clark et al. 2009). These reports suggest that RAS may be the common link between hypertension, obesity, and diabetes (Folli, Saad et al. 1999; Bernobich, de Angelis et al. 2002; Lastra-Lastra, Sowers et al. 2008). The goal of the current study is to investigate the effects of Ang II on metabolic oxidative capacity, or the ability to utilize certain substrates for energy, in skeletal muscle.

Statement of the Problem

Hypertension, obesity, and diabetes have long been linked and all are listed as a part of the metabolic syndrome (Eckel, Grundy et al. 2005). More recently, the biochemical pathways linking these conditions have been under investigation however the mechanisms behind these conditions have yet to be discerned. This study aims to investigate the role of Ang II on metabolic oxidative capacity, since a reduction in oxidative capacity has long been linked to obesity and the development of diabetes (Kelley 2005).

Significance of the Study

Currently 33% of adults and 17% of teens in the United States are classified as obese and this number is expected to continue to rise (Ogden, Yanovski et al. 2007). Obesity is associated with many comorbidities including hypertension and diabetes (Eckel, Grundy et al. 2005). The costs for treating these diseases is considerable and continues to increase; putting a significant strain on the country's health care system.

Gaining a better understanding of the pathways that link these diseases could lead to novel treatment options in the future.

Specific Aims

Skeletal Muscle Cell Culture Studies

1. To determine the effects of Ang II treatment on mammalian skeletal muscle fatty acid oxidation and neutral lipid accumulation.
2. To determine the effects of Ang II treatment on mitochondrial oxidative capacity.
3. To determine the effects of Ang II treatment on skeletal muscle glucose oxidation and glycogen synthesis.

Human Clinical Study

1. To determine the effects of blocking Ang II on muscle fatty acid oxidation.
2. To determine the effects of blocking Ang II on muscle glucose oxidation.

Main Hypotheses

Skeletal Muscle Cell Culture Studies

Treatment of cells with Ang II will increase the production of reactive oxygen species, impair mitochondrial function, and reduce oxidative capacity in skeletal muscle cells.

Human Clinical Study

Treatment of subjects exhibiting traits associated with the metabolic syndrome with Benicar® will improve oxidative capacity in skeletal muscle.

Basic Assumptions

1. The *in vivo* physiological characteristics of skeletal muscle are retained in skeletal muscle cell cultures.
2. The skeletal muscle cell line used in these studies possesses normal metabolic function.
3. The Ang II used in the cell culture studies has the identical physiological effect as Ang II naturally produced *in vivo*.
4. Benicar® is not an activator of peroxisome proliferator-activated receptor gamma (PPAR γ).

Limitations

1. L6 skeletal muscle cells derived exclusively from one tissue.
2. There are a limited number of subjects in the human clinical study.
3. Compliance of human subjects.

Chapter 2: Review of the Literature

Obesity and Metabolism

The Metabolic Syndrome

The metabolic syndrome was first documented by Swedish physician Eskil Kylin in the 1920's (Eckel, Grundy et al. 2005). Dr. Kylin noted the collection of hypertension, hyperglycemia and gout, however it was not until twenty years later that the metabolic syndrome was expanded to include type II diabetes and central obesity and given the name Syndrome X (Reaven 1988).

In 1999 the World Health Organization gave a formal definition to the disease and created an official standard for diagnosis. It is currently defined as a collection of two or more of the following symptoms: BMI greater than 30 kg/m^2 , dyslipidemia, hypertension, microalbuminuria, and insulin resistance (Eckel, Grundy et al. 2005). Recently, the prevalence of obesity, defined as a BMI greater than 30 kg/m^2 , in the United States has increased to affect approximately 33% of adults and 17% of teens (Ogden, Yanovski et al. 2007). This is creating a growing strain on the nation's health care system.

Adults with obesity are at a higher risk of developing type II diabetes and cardiovascular disease, the number one cause of death in US adults (Ogden, Yanovski et al. 2007). There is also an increasing correlation between type II diabetes in children and young adults and the rise in obesity in recent years (Eckel, Grundy et al. 2005). As such, the prevalence of research efforts focused on identifying the metabolic and cellular processes involved in obesity and type II diabetes have increased. As the understanding of these processes improves, so will treatments for these complex diseases.

Metabolic Inflexibility

Metabolic inflexibility is defined as lacking the ability to easily transition between substrates (Kelley and Mandarino 2000; Stump, Henriksen et al. 2006). Much of this also relates to a decreased tissue response to insulin. Since skeletal muscle is the most abundant insulin-sensitive tissue and is responsible for approximately 20-30% of resting oxygen consumption and 75-90% of all insulin-mediated glucose disposal, its role in the pathophysiology of the metabolic syndrome is extremely important (Baron, Brechtel et al. 1988; Zurlo, Larson et al. 1990).

Normal weight subjects, when challenged with a hyperinsulinemic-euglycemic clamp, or a meal, are able to increase the utilization and storage of glucose and markedly reduce fatty acid oxidation (Stump, Henriksen et al. 2006). Conversely, in obese and insulin-resistant individuals there is an inability to increase glucose oxidation and storage in response to hyperinsulinemia and fatty acid oxidation remains unchanged. Also, these individuals in fasted conditions demonstrate a blunted capacity for fatty acid oxidation and heightened storage compared to their lean counterparts (Storlien, Oakes et al. 2004).

Recently, it has been suggested that this increased storage of fatty acids within skeletal muscle is associated with insulin resistance (Kelley, Goodpaster et al. 1999). In extreme obesity, there is a significantly higher incorporation of fatty acids into intramuscular triglycerides (IMTG) when compared to their lean or moderately obese counterparts (Hulver, Berggren et al. 2003). These individuals also exhibit markedly lower palmitate oxidation. In this study, though there was no difference in oxidation or IMTG with moderately obese individuals, they still exhibit elevated fatty acyl CoA levels in the muscle. Many other studies have shown severely elevated plasma fatty acids in

overweight individuals, which has been suggested as one of the possible causes for insulin resistance (DeFronzo 1988; Kelley 2005). In experimental conditions, the maintenance of elevated plasma fatty acids suppresses insulin-stimulated glucose uptake by skeletal muscle as well as impairing insulin suppression of fatty acid oxidation, which mimics that seen naturally in individuals with type II diabetes and obesity (Kelley 2005). Therefore, understanding the interplay between fatty acid and glucose metabolism and the ability of insulin to effectively signal is important for development of future treatments and fully understanding the diseases associated with and caused by obesity.

Crosstalk Between Obesity and Hypertension

The Role of the Renin-Angiotensin System

A link between hypertension and obesity has been established for many years and both are a part of the metabolic syndrome. In fact, a majority of patients with type II diabetes also have hypertension (Sharma and Chetty 2005). Data from as far back as 1912 showed that an increase in BMI was associated with increasing prevalence of diabetes and hypertension and pointed out the additional problem with controlling hypertension in these patients (Sharma, Engeli et al. 2001). It has been well established that the risk of cardiovascular disease increases dramatically with increasing BMI and increased visceral fat is associated with metabolic complications of obesity, including diabetes, and hypertension (Sharma and Chetty 2005). A significant number of research efforts have been devoted to better understanding what pathways are involved in hypertension and the metabolic changes with obesity. Early studies linked hypertension

with insulin resistance, but how they fully interact is still under investigation (Lastra-Lastra, Sowers et al. 2008).

The RAS is a well-known player in blood pressure regulation. Activation of this system results in increased production of Ang II, which acts as a vasoconstrictor, thereby increasing blood pressure. Therefore, many components of this system have been targets for pharmaceutical regulation of hypertension. It was not until recently that it was implicated in the development of type II diabetes. There is impaired insulin signaling in patients with hypertension and those left untreated for hypertension exhibited higher fasting and postprandial insulin levels than their normotensive counterparts, regardless of BMI (Sowers 2004). Recently, Ang II has been recognized as the probable key player in this interplay between hypertension and insulin resistance.

Ang II uses the insulin receptor substrate (IRS) -1 to relay signals intracellularly, which at least partially explains why there is such an interplay between the two systems (Bernobich, de Angelis et al. 2002). The problem is that when Ang II binds to the Ang II receptor, the end metabolic effect is the opposite of insulin. It inhibits insulin-stimulated IRS-1 tyrosine phosphorylation, and, in part, attenuates the ability of insulin to stimulate tissue uptake of glucose by reducing glucose transporter (GLUT) 4 translocation to the cell membrane (Calegari, Alves et al. 2005; Olivares-Reyes, Arellano-Plancarte et al. 2009). Arguably, this could explain the link between hypertension, insulin resistance, and type II diabetes. Investigative efforts have recently delved into the effects of blocking components of the RAS on the onset of type II diabetes.

Effects of Blocking the RAS on Insulin Sensitivity and Diabetes

There is a growing body of evidence suggesting that insulin resistance may be a unifying factor for the development of metabolic and cardiovascular defects (Henriksen 2007). Because the RAS recently has been implicated in insulin resistance, the effect of blocking components of the RAS on insulin sensitivity and diabetes has recently been explored. Recent studies have suggested that blockade of the RAS may protect against the development of type II diabetes (Jandeleit-Dahm, Tikellis et al. 2005). This is in stark contrast to current popular hypertension treatments like diuretics and calcium channel blockers (Elliott and Meyer 2007). RAS inhibitors are currently considered the premier treatment for hypertension in patients with diabetes. However there are currently many types of inhibitors on the market. Some inhibit the angiotensin converting enzyme (ACE), which catalyzes the conversion of angiotensin I to Ang II, the active hormone. It also degrades bradykinin, a potent vasodilator (Henriksen and Jacob 2003). The other popular drug class targeting the RAS are the ARBs, which block the binding of Ang II to its receptor, thereby preventing its action. Which treatment is more effective is still not fully understood.

There have been numerous animal and clinical studies investigating the effects of ACE inhibition on insulin sensitivity and glucose disposal. Studies in obese Zucker rats showed that chronic administration of ACE inhibitors elicit an increase in whole-body insulin action and cause substantial improvements in whole-body insulin sensitivity and decreases in plasma insulin as well as an amelioration of dyslipidemia (Henriksen, Jacob et al. 1999; Henriksen and Jacob 2003). Acute administration also enhances insulin-mediated glucose transport in the skeletal muscle of obese Zucker rats.

Human clinical studies are a little less clear. The “Heart Outcomes Prevention Evaluation” (HOPE) study using the drug ramipril found patients treated with this drug saw a significant reduction in the diagnosis of new-onset diabetes compared to their peers (Yusuf, Sleight et al. 2000; Scheen 2004; Jandeleit-Dahm, Tikellis et al. 2005). However, this trial did not set out with the purpose of looking at this effect and only saw this during the post-hoc analysis. Several other trials, including the “Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial” (ALLHAT) (2002), “Captopril Prevention Project” (CAPPP) (Hansson, Lindholm et al. 1999), and “Studies of Left Ventricular Dysfunction” (SOLVD) (Vermes, Ducharme et al. 2003) found that patients treated with ACE inhibitors saw a lower incidence of new-onset diabetes than other non-RAS targeting treatments (Jandeleit-Dahm, Tikellis et al. 2005; Elliott and Meyer 2007). However not all trials have shown ACE inhibitors to be metabolically beneficial. Several studies have shown no increases in insulin sensitivity in normal subjects, those with normal insulin sensitivity, or those with type II diabetes (Henriksen and Jacob 2003). One study showed that although the obese, hypertensive subjects demonstrated a small improvement with ACE inhibition, no effects were observed in normotensive, lean subjects (Allemann, Baumann et al. 1992). Therefore, it seems as though some dysfunction in the system is necessary for ACE inhibition to have a beneficial effect.

The other popular class of RAS blocking pharmaceuticals, the ARBs, do not inhibit the formation of these compounds but instead prevent their actions by blocking the receptors. Animal studies using ARBs in the TG(mREN2)27 rats, a hypertension model that overexpresses the RAS components, shows improvements in whole-body

insulin sensitivity as well as insulin-stimulated glucose transport in isolated skeletal muscle. These results suggest that such effects cannot be completely attributed to the hemodynamic modifications occurring when blocking the RAS (Sloniger, Saengsirisuwan et al. 2005; Sloniger, Saengsirisuwan et al. 2005). These improvements were observed without any alterations in the insulin signaling cascade. Studies using obese Zucker rats show very similar results. Acute treatments of the obese rats with irbesartan, an ARB, resulted in improvements in insulin-stimulated glucose uptake in the soleus, an oxidative tissue, which was correlated significantly with the improvement in whole-body insulin sensitivity (Henriksen, Jacob et al. 2001). Chronic treatments show a reduction in circulating levels of insulin as well as improvements in several components of the insulin signaling cascade, including IRS-1 phosphorylation and Akt activation (Munoz, Argentino et al. 2006; Rizzoni, Pasini et al. 2008).

There are numerous studies that have suggested that ARBs are successful in reducing the incidence of new-onset diabetes in patients with hypertension. The “Losartan Intervention for Endpoint reduction in hypertension” (LIFE) study noted a 25% lower incidence in new-onset diabetes with the losartan treated patients (Lindholm, Ibsen et al. 2002). Similar studies of ARBs including the “Antihypertensive treatment and Lipid Profile in a North of Sweden Efficacy Evaluation” (ALPINE) (Lindholm, Persson et al. 2003), “Study on Cognition and Prognosis in the Elderly” (SCOPE) (Lithell, Hansson et al. 2003), and “Valsartan Antihypertensive Long-term Use Evaluation” (VALUE) (Julius, Kjeldsen et al. 2004) all showed reductions in the incidence of new-onset diabetes in the ARB treatment groups, though not all achieved statistical significance (Scheen 2004).

Some ARBs, such as telmisartan and irbesartan, in addition to having a positive hemodynamic effect, also activate peroxisome proliferator-activated receptor gamma (PPAR γ), a transcription factor that has many beneficial metabolic effects (Negro and Hassan 2006). The concentrations needed to achieve this effect are easily attainable through standard pharmacological dosing (Kintscher, Bramlage et al. 2007). Many trials using ARBs have shown reductions in many metabolic risk factors for diabetes and the metabolic syndrome (Mancia, Grassi et al. 2006; Kintscher, Bramlage et al. 2007). These include but are not limited to decreased in total and LDL cholesterol, as well as a decrease in triglyceride accumulation in the liver (Kyvelou, Vyssoulis et al. 2006). Since lipid metabolism is regulated by PPAR γ , it is uncertain whether these beneficial effects occur as a result of blocking the RAS or through the activation of PPAR γ . However, not all ARBs are proven PPAR agonists, so this effect is still under scrutiny. Overall, mechanisms are still unclear, but the beneficial effects are at this stage well documented.

Angiotensin II and Oxidative Stress

Recent studies have suggested that the metabolic effects of Ang II are mediated by reactive oxygen species (ROS) production, resulting in mitochondrial damage. Production of ROS has many divergent effects on cellular functions including cell growth and differentiation, mitochondrial damage, and stimulation of many kinases and proinflammatory genes (Briones and Touyz 2010). At this point in time, research is conflicting on whether the hypertension causes ROS production or visa versa, however many studies have shown that high levels of Ang II cause an increase in ROS production and that blocking this system can reverse this effect.

There are several recent studies suggesting that ROS plays an integral role in the development of Ang II induced insulin resistance. Ang II stimulates protein kinase C (PKC), causing activation of NADPH oxidase, which produces superoxide causing cellular damage (Puri, Avantiaggiati et al. 1995; Herrera, Silva et al. 2010; Phillips, Pechman et al. 2010). This activation of NADPH oxidase causes an increase in ROS production, which in many studies has been shown to disrupt the insulin signaling cascade (Wei, Sowers et al. 2006; Cooper, Whaley-Connell et al. 2007; Diamond-Stanic and Henriksen 2010). Not only does it disrupt insulin signaling, but also causes mitochondrial damage, causing changes in metabolic function including reducing fatty acid β -oxidation and disrupting the functions of several enzymes involved in oxidative metabolism (Wei, Clark et al. 2009). Recent research suggests that these metabolic changes happen as a result of activating the nuclear factor κ B (NF- κ B) pathway, resulting in an increase in inflammation, which has been shown to decrease oxidative metabolism and inhibit many oxidative metabolic enzymes (Wei, Sowers et al. 2008; Frisard, McMillan et al. 2010). Recently the RAS has been closely linked with the toll-like receptor 4 (TLR4) pathway since many of the enzymatic and protein synthesis changes are the same, resulting in similar changes in metabolism, however this needs to be further examined (Eley, Russell et al. 2008; Ji, Liu et al. 2009).

Conversely, research suggests blocking the effects of ROS reverses these effects of Ang II on metabolism and insulin signaling. If the superoxide dismutase (SOD) mimetic tempol is used to treat cells in addition to Ang II, the adverse effects of Ang II on glucose transport and insulin signaling are reversed, further supporting the notion that Ang II effects on insulin signaling are mediated by ROS production (Diamond-Stanic and

Henriksen 2010). In addition, blocking the RAS, specifically using ARBs, reduces oxidative stress and is shown to have many beneficial effects on metabolism (Wei, Sowers et al. 2008). This blockade using ARBs has also been linked to protection against mitochondrial oxidative damage and perhaps a strategy to slow ageing (De Cavanagh, Toblli et al. 2005; de Cavanagh, Flores et al. 2008; Benigni, Corna et al. 2009). Blocking Ang II actions results in increases in MnSOD, an endogenous free radical scavenger thereby reducing oxidative stress and cellular damage (Xu, Zhao et al. 2010). Overall, the interplay between the RAS and oxidative stress is intriguing but still needs to be further explored.

Angiotensin II and Skeletal Muscle

The Role in Insulin Signaling and Sensitivity

As briefly discussed earlier, there is significant crosstalk between the Ang II and insulin signaling systems. Both Ang II and insulin use the IRS pathway to elicit their intracellular effects (Folli, Saad et al. 1999). When insulin binds to the insulin receptor, it induces tyrosine phosphorylation of IRS-1, which causes downstream phosphorylation of Akt (Bjornholm and Zierath 2005). This happens as a result of PI3-kinase activity. In skeletal muscle, this activation of Akt induces GLUT4 translocation to the cellular membrane, increasing glucose uptake. However, when Ang II induces the same pathway using IRS-1, the associated PI3-kinase activity is blunted (Folli, Saad et al. 1999; Zahradka, Storie et al. 2009; Radhakrishnan, Busby et al. 2010). In studies using vascular smooth muscle cells and cardiomyocytes, Ang II pretreatment significantly reduced insulin-induced Akt phosphorylation (Motley, Eguchi et al. 2003; Ikushima, Ishii

et al. 2010). Since this is an integral part of the signal transduction to induce insulin-stimulated glucose uptake, when insulin binds in the presence of Ang II the ability of insulin to signal is diminished. One recent study suggests that in addition to the crosstalk using IRS-1, there is also a link between the systems through the suppressor of cytokine signaling-3 (SOCS-3) system (Calegari, Alves et al. 2005). SOCS-3 also promotes a reduction of IRS-1 tyrosine phosphorylation, thereby reducing the ability of insulin to signal. Though this is an interesting suggestion, as of now studies are inconclusive.

There are only a few studies investigating the effects of Ang II on insulin signaling in skeletal muscle. The TG(mREN2)27 rats, which overexpress the RAS, exhibit defective insulin signaling in skeletal muscle as well as impaired glucose tolerance and insulin sensitivity (Sloniger, Saengsirisuwan et al. 2005). In a study using L6 myotubes treated with Ang II, there was a reduction in IRS-1 phosphorylation and Akt phosphorylation as well as a reduction in GLUT4 protein expression (Wei, Sowers et al. 2006). An additional study showed that using an ARB in spontaneously hypertensive rats improved IRS-1 and Akt phosphorylation in skeletal muscle (Rizzoni, Pasini et al. 2008). These studies echo much of what is published about insulin signaling in other tissues, however there is little other research about this in skeletal muscle. One study interestingly showed that thought Ang II infusion induced insulin resistance in rats and it had this effect while actually enhancing insulin signaling (Ogihara, Asano et al. 2002). Though most data shows that Ang II induces a reduction in insulin signaling in muscle, this is an area that still needs further exploration.

Metabolic Effects

Few of the metabolic effects of Ang II on skeletal muscle are well documented. To date, the effects of Ang II on glucose uptake and utilization in skeletal muscle are conflicting. In several studies, treatment with Ang II inhibits insulin stimulated GLUT4 translocation in skeletal muscle cells (Wei, Sowers et al. 2006; Kobayashi, Akiyama et al. 2010). Treatment of the same cells with an ARB, in this case irbesartan, reversed this inhibition (Kobayashi, Akiyama et al. 2010). However, it important to note that this particular ARB is also a PPAR γ agonist, and therefore these results could be as a result of PPAR γ activation and not the blockage of RAS. In human subjects, many studies have shown positive effects on glucose uptake with ARBs, as discussed earlier, and treating obese subjects with Ang II results in a blunting of insulin stimulated glucose uptake (Dietze and Henriksen 2008; Lastra-Lastra, Sowers et al. 2008; Diamond-Stanic and Henriksen 2010). However, this blunting of insulin stimulated glucose uptake seems to only occur in overweight or hypertensive individuals. In normotensive subjects, Ang II infusion actually results in a slight increase or no change in insulin stimulated glucose uptake and carbohydrate oxidation, which is contrary to what much of the research suggests (Buchanan, Thawani et al. 1993; Townsend and DiPette 1993; Jonk, Houben et al. 2010). This suggests there may need to already be some dysfunction in the system in order for Ang II to elicit any adverse effects. Therefore, much of the effect on GLUT4 translocation and glucose uptake is still under scrutiny.

For cellular metabolism data, there is little research out there on the effects on Ang II on skeletal muscle substrate use or changes in oxidation. Though much can be hypothesized based on results from other tissue types or inferences based on what is

known about the cellular pathways, actual data are sparse. In human subjects, most of the changes in metabolism are suggested to be a result of hemodynamic mechanisms, and not necessarily direct changes in cellular signaling (Buchanan, Thawani et al. 1993; Richey, Ader et al. 1999; Chai, Wang et al. 2010). Also, only a couple of studies measure substrate oxidation changes, and none are in human subjects. Because Ang II is linked with oxidative stress and mitochondrial damage, it is logical to assume that some of the metabolic pathways occurring in the mitochondria may suffer adverse effects, however there are only a couple of studies exploring these effects. One study using the TG(mREN2)27 rats showed a significant reduction in fatty acid β -oxidation, as well as reduction in enzyme activity of β -hydroxyacyl-CoA-dehydrogenase (β HAD), an enzyme involved in this pathway, and activity of citrate synthase, the rate limiting step in the tricarboxylic acid cycle (TCA) (Wei, Sowers et al. 2008). A similar study using C57bl/6 mice showed very similar results. Those animals infused with Ang II showed higher storage of triglycerides, and significant reductions in fatty acid β -oxidation and β HAD activity (Mitsuishi, Miyashita et al. 2009). Currently, no human studies have explored these effects and as such, more research is necessary in this area.

Summary

The prevalence of obesity, defined as a BMI greater than 30 kg/m^2 , in the United States has increased to affect approximately 33% of adults and 17% of teens (Ogden, Yanovski et al. 2007). Obesity is associated with many comorbidities including hypertension, diabetes, and cardiovascular disease, the leading cause of death in the United States. The crosstalk between cardiovascular diseases, including hypertension,

and obesity has been well investigated and the link that seems the strongest is the RAS, specifically Ang II (Bernobich, de Angelis et al. 2002; Sharma and Chetty 2005).

Skeletal muscle of obese individuals exhibit reduced oxidative capacity and an inability to transition between substrates, much of which is linked to insulin resistance (Kelley, Goodpaster et al. 1999; Hulver, Berggren et al. 2003; Storlien, Oakes et al. 2004). Since Ang II shares many of the intracellular signaling molecules with the insulin signaling system, and has been linked with insulin resistance, it is now being investigated as one of the major players in the link between hypertension and the metabolic changes associated with obesity and diabetes (Folli, Saad et al. 1999; Bernobich, de Angelis et al. 2002). Blocking the RAS has been effectively shown to decrease new-onset diabetes and improve insulin sensitivity, but many of the metabolic effects of Ang II, and the metabolic effects of blocking this system, have not been thoroughly investigated as of yet (Henriksen, Jacob et al. 2001; Scheen 2004; Sloniger, Saengsirisuwan et al. 2005).

Chapter 3: Methods

Cell Culture Studies

Skeletal Muscle Cell Lines

Cell culture studies were conducted using L6 rat skeletal muscle cell line. Cells were purchased from the American Type Culture Collection (Manassas, VA) and grown to ~80% confluence in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin, (50 µg/ml) (Invitrogen, Carlsbad, CA) and sub-cultured in either 6 or 12-well plates (Becton Dickinson, Franklin Lakes, NJ). Cells were grown to confluence and then differentiated into myotubes in DMEM containing 2% horse serum, penicillin (50 U/ml), and streptomycin (50 µg/ml) (Invitrogen, Carlsbad, CA). All experiments were performed between days 5-7 of differentiation.

Angiotensin II Treatments

At 5-7 days of differentiation, L6 cells were treated with human Ang II peptide (Sigma Chemicals, St. Louis, MO) at a concentration of 10^{-7} M for 3 or 24 hours, unless otherwise noted, and glucose oxidation, glycogen synthesis, and fatty acid metabolism will be assessed. The effects of Ang II on ROS production were assessed in a time course study in which cells were imaged via confocal microscopy immediate post treatment (minutes), or at 1, 2, and 3 hours post treatment. For western blot analysis, after 24 hour Ang II treatment, cells were treated with insulin (10^{-7} M) for 10 minutes to look at components of the insulin signaling cascade.

Fatty Acid Metabolism

Fatty acid oxidation medium contained 0.5 $\mu\text{Ci/mL}$ of $[1-^{14}\text{C}]$ -palmitate (Perkin Elmer, Waltham, MA), 100 μM palmitic acid, 0.25% bovine serum albumin (Sigma Chemicals, St. Louis, MO), 1mM carnitine (Sigma Chemicals, St. Louis, MO), and 12.5mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) (Sigma Chemicals, St. Louis, MO) and brought to volume in starvation media containing high glucose DMEM, penicillin (50 U/ml), and streptomycin (50 $\mu\text{g/ml}$). Before addition of starvation media, carnitine, and (HEPES), both cold and $[1-^{14}\text{C}]$ -palmitate were pre-incubated for 30 minutes at 37°C.

Fatty acid oxidation medium, 0.5 mL per well, was added to 12-well plates sealed with parafilm, and incubated for 3 hours at 37°C. After the incubation period the cells were harvested as previously described (Muioio, MacLean et al. 2002; Hulver, Berggren et al. 2005). Palmitate oxidation was measured using previously described procedures (Hulver, Berggren et al. 2003). Phospholipid, free fatty acid, monoacylglycerol, diacylglycerol, and triacylglycerol production was measured by thin layer chromatography as previously described (Hulver, Berggren et al. 2003).

Glucose Metabolism

Glucose oxidation medium contained 1 $\mu\text{Ci/mL}$ of $[\text{U}-^{14}\text{C}]$ -glucose (Perkin Elmer, Waltham, MA), 12.5mM HEPES (Sigma Chemicals, St. Louis, MO) and brought to volume in starvation media containing high glucose DMEM, penicillin (50 U/ml), and streptomycin (50 $\mu\text{g/ml}$).

Glucose oxidation medium, 0.5 mL per well, was added to 12-well plates sealed with parafilm, and incubated for 3 hours at 37°C. After the incubation period the cells were harvested as previously described (Muio, MacLean et al. 2002; Hulver, Berggren et al. 2005). Total oxidation was measured using previously described procedure (Hulver, Berggren et al. 2005).

Glycogen Synthesis

Prior to incubation in the glycogen media, cells were pre-incubated in Kreb's Ringer Buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO₄• 7 H₂O, 1.2 mM CaCl • 2 H₂O, 20 mM HEPES) (Sigma Chemicals, St. Louis, MO) for 5 hours at 37°C. Glycogen synthesis media containing 5mM cold glucose (Sigma Chemicals, St. Louis, MO), 1.25 μCi/mL of [U-¹⁴C]-glucose (Perkin Elmer, Waltham, MA), and 10⁻⁷M insulin in Kreb's Ringer buffer was added to the cells and incubated for 3 hours at 37°C. After incubation, media was aspirated and cells solubilized using 0.25 mL of 0.2 M NaOH (Sigma Chemicals, St. Louis, MO) per well and scraped on ice. Solubilized cells were collected in 2mL eppendorf tubes containing 35 uL of 60mg/mL glycogen (Sigma Chemicals, St. Louis, MO). Tubes were then incubated for 20 minutes on an 80°C heat block. Samples were then cooled on ice and 0.5 mL of cold 100% ethanol (Fisher Scientific, Pittsburgh, PA) was added to the tubes to precipitate glycogen. Tubes were then centrifuged at 10,000rpm for 20 minutes at 4°C to pellet the glycogen. Supernatant was removed and pellet was washed with 70% ethanol (Fisher Scientific, Pittsburgh, PA) then spun again at 10,000rpm for 10 minutes. Supernatant was removed and pellets allowed to air dry. Pellets were then resuspended in 0.5mL of deionized water, after which sample was

placed in a scintillation vial and scintillation fluid added. Samples were then counted on a LS-6500 Liquid Scintillation Counter (Beckman Coulter, Brea, CA) to quantify the ^{14}C -labeled glycogen in each sample. This data was corrected to protein content for all samples.

Mitochondrial Respiration

Mitochondrial respiration of L6 skeletal muscle cells was performed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA) as described by Choi et al with modifications (Gerencser, Neilson et al. 2009). Cells were seeded into 20 well XF24 V7 cell culture microplates at a density of 5,000 cells per well. Cells were grown unto 80% confluence and differentiated into fully differentiated myotubes. Experiments were conducted in serum free media on day 7 of differentiation. Immediately following treatments, cells were loaded into the XF24. Experiments consisted of 3 minute mixing, 2-minute wait, and 2-minute measurement cycles, unless otherwise stated. Oxygen consumption was measured under basal conditions, in the presence of the mitochondrial inhibitors oligomycin (0.5 μM) or rotenone (0.25 μM), or in the presence of the mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (0.3 μM) to assess maximal oxidative capacity. All experiments was performed at 37 °C.

Confocal Imaging of Reactive Oxygen Species Production

Intracellular ROS levels was measured in L6 skeletal muscle cells using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes, Invitrogen, Carlsbad, CA) as previously described with modifications (Palomero, Pye et al. 2008). Cells were incubated with 10 μ M of 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H₂DFFDA, Invitrogen, Carlsbad, CA) for 30 minutes at 37°C prior to imaging. Cells were then washed and exposed for 400 ms and allowed to rest for 15 minutes post exposure. Cells were imaged immediately post treatment with Ang II, after 1 hour, 2 hours, and 3 hours and imaged once per minute over a 5 minute time period to measure DCFDA fluorescence. Imaging for intracellular ROS levels was conducted on a Zeiss LSM 510 Meta microscope (Carl Zeiss, Oberkochen, Germany) with a 25x water immersion objective. Images were taken using a 488 nm excitation filter and a 500 nm long pass emission filter.

Western Blot

Western analysis was performed using cell lysates harvested in Mammalian Cell Lysis Buffer (Sigma Aldrich, St Louis, MO). Proteins were separated using a 10% Criterion-Tris- HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to PVDF membrane (Bio-Rad, Hercules, CA). Blots were probed with primary antibodies against β -actin (Cell Signaling, Danvers, MA; 1:1000), total-Akt (Cell Signaling, Danvers, MA; 1:1000), phosphorylated-Akt (Cell Signaling, Danvers, MA; 1:1000), and SOD-2 (Abcam, Cambridge, MA; 1:1000) followed by anti-rabbit or mouse secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA; 1:10,000). Proteins were

visualized using Super-Signal Chemiluminescent Substrate (Pierce, Rockville, IL) and a ChemiDoc XRS Imaging System (BioRad, Hercules, CA).

qRT-PCR mRNA Analysis

RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. qRT-PCR was performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to manufacturer's specifications (Applied Biosystems, Inc., Foster City, CA). Target gene expression was normalized to β -actin RNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as pre-validated assays (Applied Biosystems, Inc., Foster City, CA). Relative quantification of target genes was calculated using the $2^{-\Delta\text{CT}}$ method, which was validated for each primer/probe set using a 6 point serial standard curve as described previously (Way, Harrington et al. 2001). Derivation of the $2^{-\Delta\text{CT}}$ equation has been described in Applied Biosystems User Bulletin No. 2 (P/N 4303859).

Statistical Analysis

Results were analyzed with 2-tailed Student's t tests or 2-way ANOVA with Tukey's post-hoc tests (multiple comparisons). Results are expressed as mean \pm SEM. The level of significance is set at $p < 0.05$.

Human Clinical Study

Subjects

Eligible candidates were males or females between 18 and 75 years of age. Females were postmenopausal and not receiving hormone replacement therapy. Subjects had a BMI greater than or equal to 25 kg/m^2 or a measured body fat of at least 20% for men and 25% for women. In addition, they had blood pressure greater than or equal to 120/80 mmHg but less than 160/100 mmHg. Factors for exclusion from the study included diabetes, secondary hypertension (hypertension with a known cause), a history of stroke, myocardial infarction or chronic kidney disease (or renal artery stenosis), or cardiovascular (e.g., chronic heart failure), respiratory (e.g., chronic obstructive pulmonary disease), neurological (e.g., Parkinson's), metabolic (e.g., hyperthyroidism), oncological (e.g., active cancer) diseases or other diseases that would make participation unsafe. All participants were approved by the medical director of the study, Dr. Jose Rivero.

Treatments

Study was a crossover design with all subjects receiving Benicar® treatment and no treatment as a control. Which treatment was given first was determined randomly. During the Benicar® treatment period, subjects took one 20 mg Benicar® pill every day for 2 weeks and then two 20 mg pills (40 mg of Benicar®) for another 6 weeks. If a subject's blood pressure fell below 110/70 mmHg during the first two weeks, then they continued to take only one pill each day for the remainder of the study. They continued

to take Benicar® during all of the follow-up measurements, which took approximately 2 weeks. Subjects were asked to not change daily diet and physical activity throughout the entire study. Between treatment periods, there was a 2-week period before the subject began the next treatment period. Before and after each treatment period, subjects came in for follow up testing, where muscle biopsies were taken along with other tests not included in this document.

All muscle biopsies were taken from the vastus lateralis muscle on either leg, depending on the subject's preference. Site was cleansed with Betadine® and a sterile, fenestrated drape placed over the site. A localized numbing agent, Lidocaine®, was administered to the skin and into the muscle tissue prior to the procedure. A small incision (approximately ¼ inch) was made into the skin and muscle fascia for the biopsy needle to pass through. The biopsy needle was then be inserted into the incision and using a small amount of suction, a small sample (50-100mg) of tissue was taken. Several passes were performed to get enough tissue for analysis. After the procedure was completed, the incision was cleaned and covered with gauze and a clear dressing. Ice was applied using an ACE® bandage to minimize swelling.

Fatty Acid Oxidation

Fatty acid oxidation was assessed in vastus lateralis muscle biopsy sample homogenates by measuring and summing $^{14}\text{CO}_2$ production and ^{14}C -labeled acid-soluble metabolites from the oxidation of [1- ^{14}C]-palmitic acid as previously described (Hulver, Berggren et al. 2005).

Glucose Oxidation

Glucose oxidation was assessed in vastus lateralis muscle biopsy sample homogenates by measuring $^{14}\text{CO}_2$ production from the oxidation of [U- ^{14}C]-glucose (Perkin Elmer, Waltham, MA) as previously described (Hulver, Berggren et al. 2005) with the exception that glucose was substituted for BSA-bound palmitic acid.

Enzyme Activity

Enzyme activities were assessed in muscle homogenates (20-fold dilution). Sample buffer consists of 0.1 mol/l $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ and 2 mmol/EDTA, pH 7.2. Phosphofructokinase, citrate synthase, and malate dehydrogenase activities were determined spectrophotometrically as previously described (Heilbronn, Civitarese et al. 2005).

Statistical Analysis

A repeated measures analysis of variance was used to test the impact of Benicar® treatment on the primary outcome. The model included sequence, subject within sequence, period, and treatment as factors. The sequence effect was tested using subjects within sequence effect as the error term. The treatment and period effects were tested against the residual mean square error. Paired t-tests were used to compare changes in the primary outcome in the Benicar® treatment and no treatment groups. Results are expressed as mean \pm SEM. The level of significance is set at $p < 0.05$.

Chapter 4: Results

Cell Culture Results

24 Hour Ang II Pre-treatment Does Not Affect Fatty Acid or Glucose Oxidation

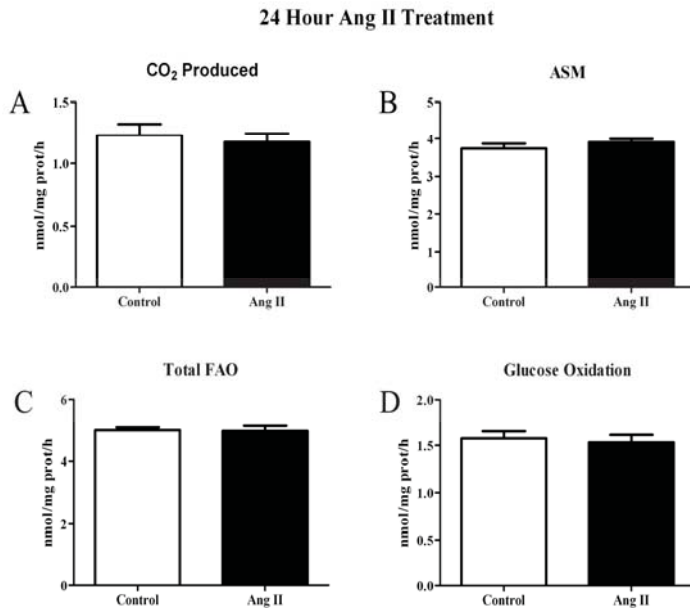


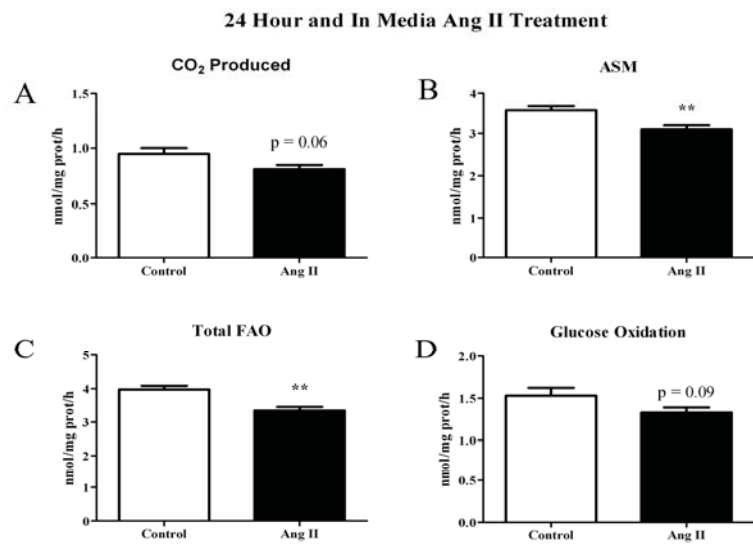
Figure 1: Ang II Effects on Oxidation After 24 Hour Pre-treatment Cells were treated for 24 hours with an Ang II pretreatment then treatment media removed for the oxidation experiments. Treatment resulted in no significant changes in fatty acid or glucose oxidation.

Treatment of cells with Ang II for 24 hours then removing it from the media for oxidation experiments had no effect on fatty acid or glucose oxidation (Figure 1). There was no significant effect on either CO₂ production or ASM production, and therefore no effect on overall fatty acid oxidation.

24 Hour and In Media

Treatment with Ang II Reduces Fatty Acid and Glucose Oxidation

Treatment of cells with Ang II both for 24 hours and in the oxidation medium during the



** p<0.01 vs. Control

Figure 2: Ang II Effects on Oxidation After 24 Hour Treatment in Oxidation Media Cells were treated with Ang II for 24 hours and treatment remained in the oxidation media for the duration of the experiment. This resulted in a non-significant ($p=0.06$) trend for decreased overall fatty acid oxidation (A). Also, significant decreases in ASM (B) ($p=0.009$) and total CO₂ (C) ($p=0.004$) were observed. In addition, there is a non-significant ($p=0.09$) reduction in glucose oxidation (D).

oxidation experiments resulted in reductions in both fatty acid and glucose oxidation (Figure 2). Ang II-induced decreases in CO₂ production from palmitate oxidation trended toward statistical significance (p=0.06) (Figure 2A), while both ASM production and total fatty acid oxidation reached p values less than 0.05 (Figure 2B and C, respectively). Ang II treatment resulted in a non-statistically significant reduction in glucose oxidation (p=0.09) (Figure 2D).

Ang II Present in the Oxidation Media Reduces Total Fatty Acid Oxidation and Increases Glucose Oxidation

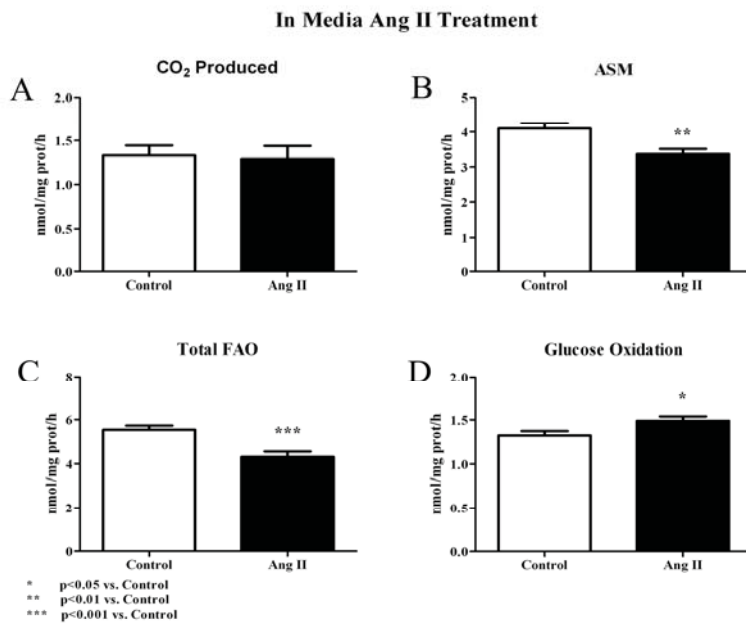


Figure 3: Ang II Effects on Oxidation Without Pre-treatment Cells were treated in Ang II exclusively during the oxidation experiments (3 hours) without pretreatment. This resulted in no change in complete palmitate oxidation (A). However, significant reductions in acetyl-CoA (B) ($p=0.01$) and total (C) ($p=0.001$) palmitate oxidation. In addition, there is a significant ($p=0.05$) increase in glucose oxidation (D).

Ang II present in the oxidation media without pretreatment resulted in no change in CO₂ production from fatty acid oxidation (Figure 3A). However, there were significant reductions in ASM production (p<0.01) (Figure 3B) and total fatty acid oxidation (p<0.001)

(Figure 3C). In addition, there was a significant (p<0.05) increase in glucose oxidation with Ang II treatment (Figure 3D).

Ang II Does Not Affect Neutral Lipid Synthesis

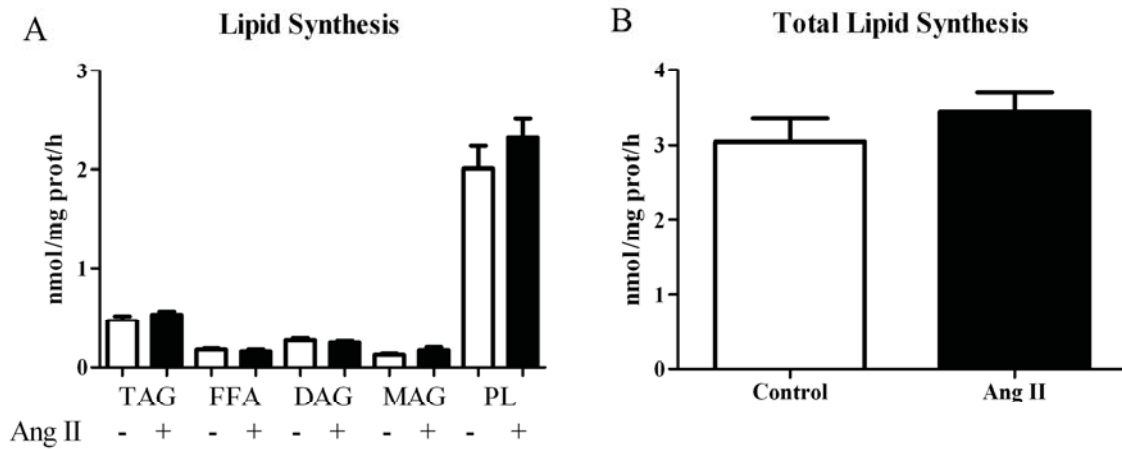


Figure 4: Ang II Effects on Neutral Lipid Synthesis Cells were incubated in fatty acid oxidation medium containing radiolabeled palmitate with or without Ang II for 3 hours. This resulted in no changes in lipid synthesis for any of the individual lipid fractions (A) or in total lipid synthesis (B).

Ang II treatment for three hours had no significant effect on total lipid synthesis (Figure 4B). In addition, there were no significant differences in any of the individual lipid fractions (Figure 4A).

Ang II Does Not Affect Insulin Stimulated Glycogen Synthesis

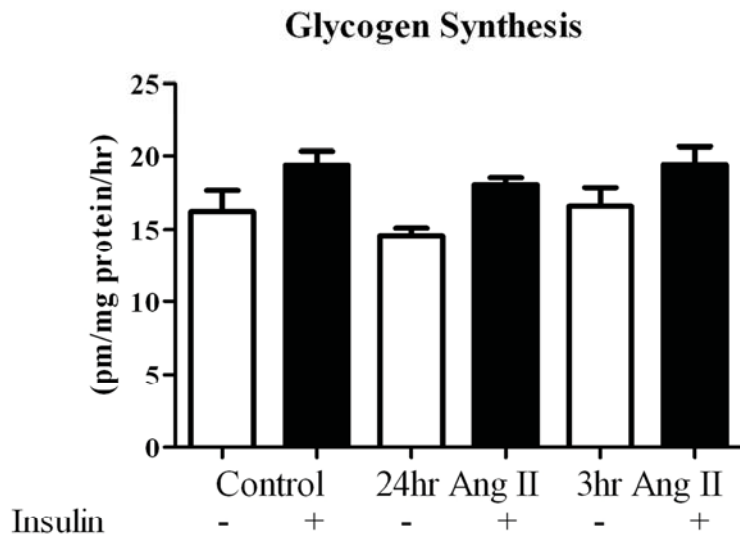


Figure 5: Ang II Effects on Glycogen Synthesis Cells were incubated in glycogen synthesis media containing radiolabeled glucose for 24 or 3 hours with or without Ang II or insulin stimulation. This resulted in no change in basal or insulin stimulated glycogen synthesis at either time point.

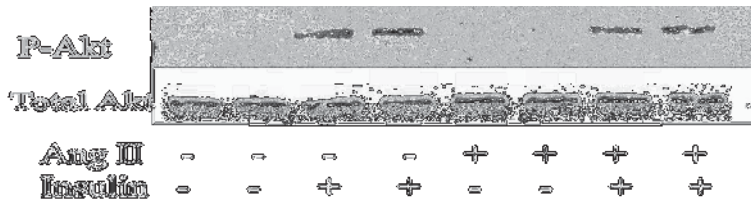


Figure 6: Ang II Effects on Insulin Signaling Cells were treated with Ang II for 3 hours with or without insulin stimulation for 30 minutes post treatment. Protein was extracted and blots probed for phosphorylated-Akt. Ang II treatment resulted in no change in insulin stimulated Akt phosphorylation.

Ang II treatment for either 24 or 3 hours while present in the glycogen synthesis media had no effect on

glycogen synthesis (Figure 5). No effect was seen either at baseline or after insulin stimulation. In addition, Ang II had no effect on insulin stimulated Akt phosphorylation (Figure 6).

Ang II Significantly Reduces Mitochondrial Maximal Oxygen Consumption

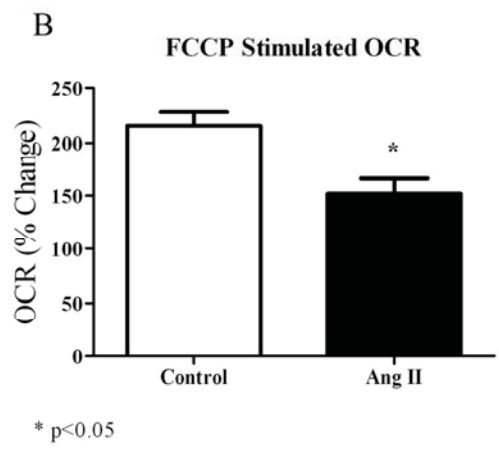
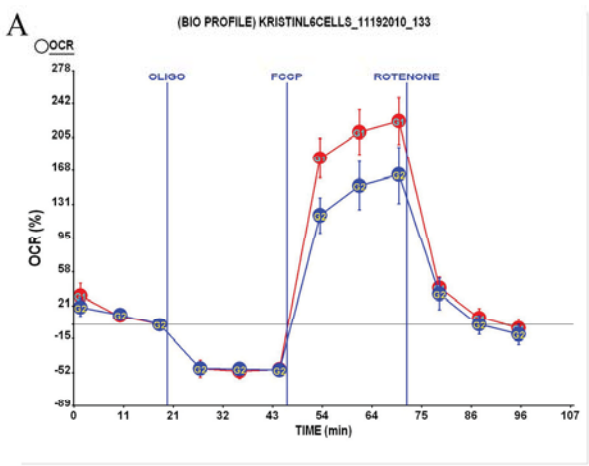


Figure 7: Ang II Effects on Mitochondrial Oxygen Consumption Cells were treated with Ang II for 24 hours then mitochondrial oxygen consumption measured using an XF 24 Flux Analyzer (Seahorse Bioscience). Treatment with Ang II significantly ($p = 0.024$) reduced maximal oxygen consumption (B), increased oxygen consumption after treatment with FCCP, a mitochondrial uncoupler.

Ang II treatment significantly ($p < 0.05$) reduced maximum oxygen consumption rate (OCR) as measured in the presence of FCCP, a mitochondrial uncoupler (Figure 7B). No differences in basal or ADP-stimulated respiration were observed with Ang II treatment (Figure 7A).

Ang II Significantly Reduces mRNA Expression of DRP1

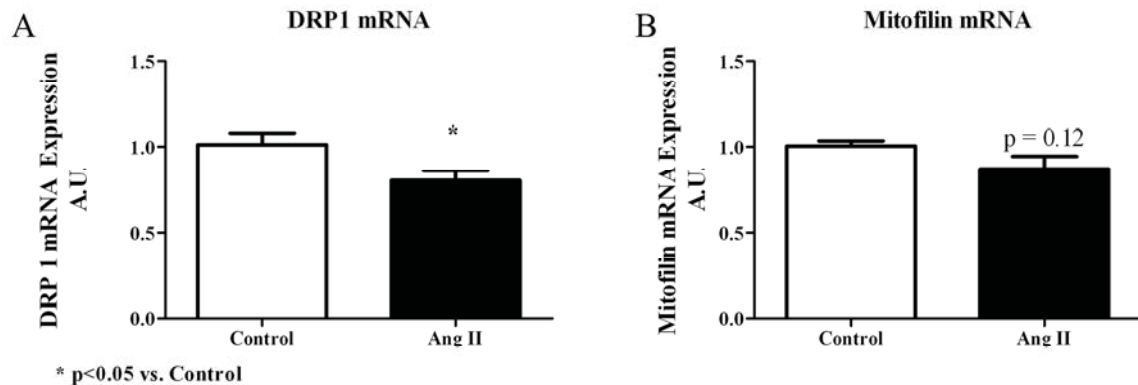


Figure 8: Ang II reduces the mitochondrial protein DRP1 and its mRNA expression. Cells were treated with Ang II for 3 hours then mRNA was extracted. Ang II treatment resulted in a significant ($p < 0.05$) reduction in DRP1 mRNA expression (A) and a non-significant ($p = 0.12$) reduction in mitofilin mRNA expression (B).

Ang II treatment for three hours significantly ($p < 0.05$) reduced mRNA levels of dynamin related protein 1 (DRP1), a protein necessary for mitochondrial fission (Figure 8A). In addition, it also reduced mitofilin mRNA in a non-significant ($p = 0.12$) but trending fashion (Figure 8B). However, Ang II treatment for three hours had no effect on mRNA levels of ATP synthase, mitofusin 1 & 2, OPA1, UCP3, or DNMI1 (Figure 9).

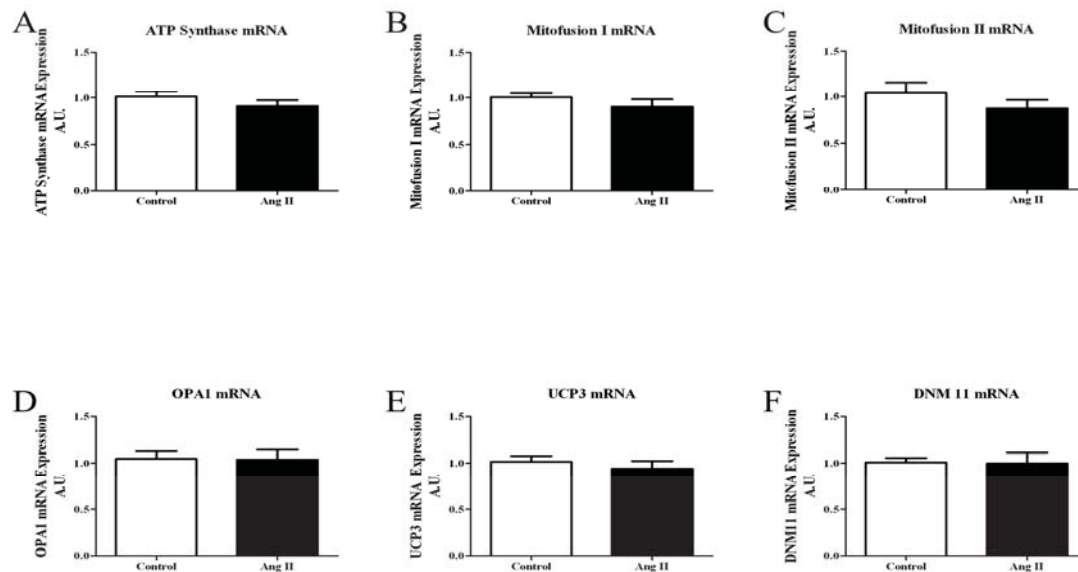


Figure 9: Ang II Effects on Mitochondrial Genes Cells were treated with Ang II for 3 hours then mRNA was extracted. Ang II treatment resulted in no significant change in ATP synthase (A), Mitofusion I (B), Mitofusion II (C), OPA1 (D), UCP3 (E), or DNMI1 (F) expression.

Ang II Treatment Increases ROS Production But Does Not Affect Expression of Cellular Antioxidants

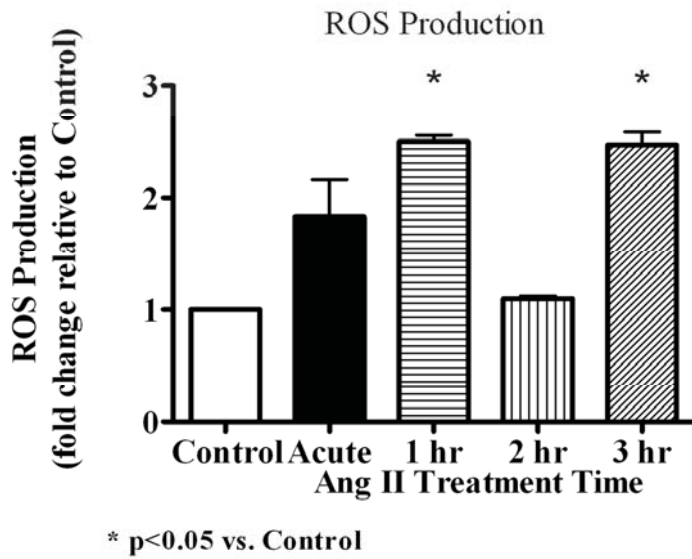


Figure 10: Ang II Effects on ROS Production Cells were treated over a 3 hours timecourse and ROS measured using confocal microscopy. Ang II resulted in no significant change in ROS production acutely or at 2 hours, however there were significant increases in ROS production at 1 hour and 3 hours.

Ang II treatment significantly increased ROS production after 1 and 3 hour treatments, however there was no effect with an acute treatment or after 2 hours (Figure 10). Despite changes in ROS production, Ang II treatment for three hours

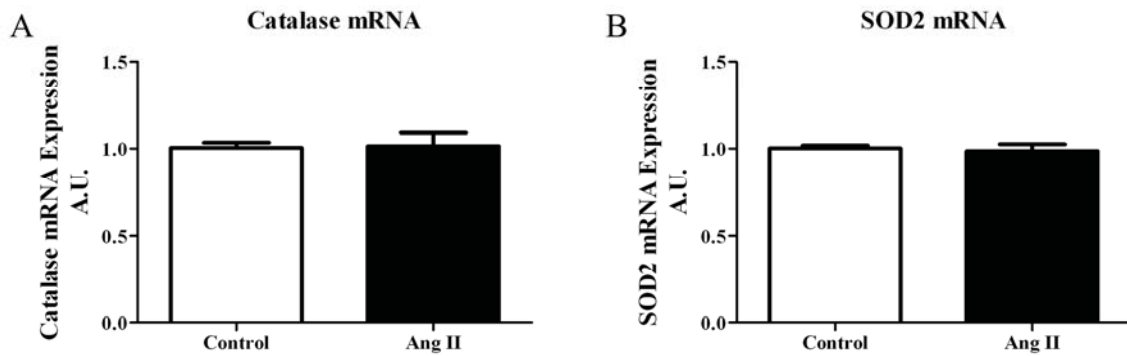


Figure 11: Ang II Effects on Cellular Antioxidants Cells were treated for 3 hours with Ang II and mRNA extracted. Ang II treatment resulted in no change to cellular antioxidant mRNAs (A) or SOD2 (B).

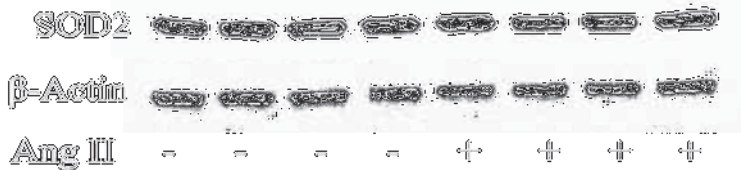


Figure 12: Ang II Effects on SOD2 Protein Cells were treated with Ang II for 3 hours then blots probed for SOD2 protein expression. Ang II treatment resulted in no change in SOD2 protein.

had no effect on mRNA expression of catalase or SOD2, two important cellular antioxidants

(Figure 11). In addition, Ang II treatment had no effect on protein expression of SOD2 (Figure 12).

Human Clinical Study Results

Treatment	Fatty Acid Oxidation (nmol/mg protein/hr)			Glucose Oxidation (nmol/mg protein/hr)	Enzyme Activity (nmol/mg protein/min)		
	CO ₂	ASM	Total	CO ₂	Citrate Synthase	Malate Dehydrogenase	PFK
Pre-ARB	1,241 ± 0.270	10,386 ± 2,534	11,626 ± 2,749	3,445 ± 1.116	111,067 ± 16,674	222,128 ± 34,538	30,332 ± 7,031
Post-ARB	1,740 ± 0.295	17,914 ± 5,157	19,654 ± 5,068	5,652 ± 0.846	94,834 ± 10,773	269,121 ± 34,725	54,005 ± 19,084
Pre- Control	1,392 ± 0,559	9,797 ± 1,949	11,189 ± 2,163	2,876 ± 1,018	88,027 ± 9,339	207,594 ± 20,698	38,745 ± 6,513
Post- Control	2,013 ± 0,417	13,162 ± 2,164	15,175 ± 2,489	5,393 ± 1,042	86,901 ± 6,144	222,583 ± 29,075	44,935 ± 4,210

Table 1: Human Clinical Study Data Muscle biopsies from the vastus lateralis were taken before and after each treatment period. Samples were homogenized and incubated in either ¹⁴C-palmitate or glucose for 1 hour then measures taken for complete and incomplete oxidation. Samples were also used to measure enzyme activity of TCA cycle enzymes citrate synthase and malate dehydrogenase and glycolysis enzyme phosphofructokinase (PFK). There were no significant differences in any of the treatment groups for any of these measures. Oxidation data expressed as nmol/mg protein/hr and enzyme activity expressed as nmol/mg protein/min. All data expressed as mean ± SEM.

The human clinical study yielded no significant changes in fatty acid or glucose oxidation. There were also no significant changes in enzyme activity of citrate synthase, malate dehydrogenase, or phosphofructokinase (PFK).

Chapter 5: Discussion

Introduction

The role of Ang II in obesity and diabetes is a new and growing body of research. Since a majority of patients with type II diabetes also have hypertension, a large amount of recent research has examined the pathways linking the two (Sharma and Chetty 2005). Ang II, being one of the major players in blood pressure regulation, has recently been implicated in the development of diabetes (Sowers 2004). Ang II is strongly linked with decreased insulin signaling and insulin resistance (Bernobich, de Angelis et al. 2002; Olivares-Reyes, Arellano-Plancarte et al. 2009). In addition, blockade of the RAS has been shown in human subjects to improve insulin sensitivity and decrease the instance of new onset diabetes (Henriksen and Jacob 2003; Jandeleit-Dahm, Tikellis et al. 2005; Sharma and Chetty 2005; Henriksen 2007). However, the role Ang II plays in skeletal muscle metabolism has received little examination.

The TG(mREN2)²⁷ rats, a hypertension model overexpressing the RAS, exhibit defective insulin signaling in skeletal muscle as well as impaired glucose tolerance and insulin sensitivity (Sloniger, Saengsirisuwan et al. 2005). Treatment of L6 cells with Ang reduced IRS-1 phosphorylation and Akt phosphorylation as well as GLUT4 protein expression (Wei, Sowers et al. 2006). Several studies show treatment with Ang II inhibits insulin stimulated GLUT4 translocation in skeletal muscle cells (Wei, Sowers et al. 2006; Kobayashi, Akiyama et al. 2010). One study using the TG(mREN2)²⁷ rats showed a significant reduction in β -oxidation as well as decreased activity of β HAD and citrate synthase, two oxidative enzymes (Wei, Sowers et al. 2008). A similar study using C57bl/6 mice showed very similar results. Those animals infused with Ang II showed

higher storage of triglycerides, and significant reductions in fatty acid β -oxidation and β HAD activity (Mitsuishi, Miyashita et al. 2009). There are currently no studies investigating the effects on oxidative metabolism in humans.

Major Findings

The major findings of these studies are: (1) Ang II reduced mitochondrial oxidative capacity after 24 hour treatment with Ang II present in the media; (2) presence of Ang II in the oxidation media reduced fatty acid oxidation; (3) Ang II had no effect on insulin stimulated glycogen synthesis or Akt phosphorylation after either 3 or 24 hours of treatment; (4) Ang II caused a significant increase in ROS production after 1 and 3 hours.

Ang II Effects on Fatty Acid Oxidation and Lipid Synthesis

Previous research suggested that high levels of Ang II decrease β -oxidation in animals, however there have been no cell culture studies focused on potential the mechanism(s) behind this effect (Wei, Sowers et al. 2008; Mitsuishi, Miyashita et al. 2009). The results presented herein show that Ang II reduced β -oxidation in L6 skeletal muscle cells, however this effect was only present when Ang II was included in the reaction media at the time substrate oxidation was measured (Figures 2 and 3). When Ang II was used exclusively for a 24 hour pre-treatment without inclusion in reaction media during measures of metabolism, no effect was observed on either complete or incomplete fatty acid oxidation (Figure 1A and B). Interestingly, the strongest reduction in complete oxidation to CO_2 was seen when cells were pretreated with Ang II for 24

hours and also present in the media during substrate oxidation measures (Figure 2A). Incomplete oxidation to ASMs was reduced when Ang II was present in the media, regardless of treatment length (Figures 2B and 3B). Interestingly, there was a much stronger reduction in total FAO in the cells treated only for a short time, however this seems to be driven by reductions in incomplete oxidation, as there was no significant change in complete oxidation to CO₂ (Figure 3C). This suggests that there is no change in cellular oxidative capacity, especially since there is an increase in glucose oxidation at this time. It is possible that these changes in total fatty acid oxidation may be driven by a reduction in β -oxidation flux, as ASMs produced during fatty acid oxidation are thought to be markers of β -oxidation. In addition, there was a significant reduction in overall mitochondrial oxidative capacity with 24 hour Ang II treatment, measured as oxygen consumption rate (Figure 7). Therefore, these data suggest some mitochondrial dysfunction as a result of Ang II treatment, though the mechanisms are currently unclear.

Although there are changes in fatty acid oxidation with Ang II treatment at three hours, there were no significant changes in neutral lipid synthesis (Figure 4). However, since the most significant effects on oxidation and oxidative capacity were seen with a 24 hour treatment with Ang II in the media, looking at neutral lipid synthesis at this time would be interesting.

Ang II Effects on Glucose Oxidation, Insulin Signaling, and Glycogen

Synthesis

Many studies have investigated the divergent effects of Ang II on insulin signaling and glucose uptake, however there are few have studied glucose oxidation or

glycogen synthesis. The results presented herein show that, like fatty acid oxidation, a 24 hour pre-treatment had no effect on glucose oxidation (Figure 1D). When cells were treated for 24 hours and Ang II left in the media during oxidation measures, there was a non-significant reduction in glucose oxidation (Figure 2D). Interestingly, the effect was the opposite with a 3 hour Ang II treatment with no pre-treatment. In this case, there was a significant increase in glucose oxidation with a reduction in fatty acid oxidation (Figure 3D). This is much like the phenotype seen in obese humans, mice, and cell lines (Kelley, Goodpaster et al. 1999; Hulver, Berggren et al. 2003; Hulver, Berggren et al. 2005; Frisard, McMillan et al. 2010). This more glycolytic, less oxidative state in muscle has been linked to reduced responsiveness to insulin and metabolic inflexibility (Simoneau and Kelley 1997; Kelley and Mandarino 2000).

Unlike glucose oxidation, there was no effect of Ang II on glycogen synthesis, with or without insulin stimulation under any condition (Figure 5). In addition, there was no effect on insulin stimulated Akt phosphorylation (Figure 6). This result is in contrast to the results seen by Wei, et al in 2006 and 2008 in the same cell type. In both papers, this group saw a significant reduction in insulin stimulated Akt phosphorylation with a 24 hour Ang II treatment at the same concentration (Wei, Sowers et al. 2006; Wei, Sowers et al. 2008). The discrepancy between the results reported here and those of Wei et al. is not clear at this time. Many human studies using obese individuals have seen the same results on a reduction in insulin stimulated Akt phosphorylation and a reduction in insulin sensitivity, as well as a decrease in glucose uptake (Dietze and Henriksen 2008; Lastra-Lastra, Sowers et al. 2008; Diamond-Stanic and Henriksen 2010). However, several studies have shown that treatment of normotensive or lean subjects with Ang II has no

effect or in some cases, caused an increase in glucose uptake (Buchanan, Thawani et al. 1993; Townsend and DiPette 1993; Jonk, Houben et al. 2010). These data suggest that Ang II has little to no effect in healthy, nonobese humans and a deleterious effect of Ang II is only observed in a state of metabolic derangement, as observed with obesity or metabolic syndrome. Perhaps if such a scenario were recapitulated in cell culture (i.e., combination treatments with Ang II and moderate to high levels of free fatty acids), the effects of Ang II on insulin signaling would be more robust.

Ang II Effects on Mitochondrial Genes

Since there were reductions in oxygen consumption with with Ang II treatment, it is possible that there are some mitochondrial changes driving these effects. Mitochondria undergo constant changes involving fission and fusion. Several studies have reported mitochondrial dysfunction and changes in mitochondrial dynamics with obesity and type II diabetes (Zorzano, Liesa et al. 2009; Zorzano, Liesa et al. 2010). Ang II treatment for 3 hours significantly reduced mRNA expression of DRP1, which is a protein that plays an integral role in mitochondrial fission (Figure 8A). However, there was no effect on the related gene DNM 11, also involved in mitochondrial fission (Figure 9F). In addition, there was no effect on expression of mitofilin, a gene involved in cristae morphology (Figure 8B).

Studies have shown that reductions in or repression of mitofusion 2 cause significant reductions in glucose and palmitate oxidation, as well as significant reductions in oxidative capacity (Zorzano, Liesa et al. 2009). The results of this thesis study showed no change in mitofusion I and mitofusion II (Figure 9B and C). There was also no

change in OPA1, another gene involved in mitochondrial fusion (Figure 9D). However, these gene results were after only a 3 hour treatment, so it is possible that the Ang II treatment must be longer to elicit this effect.

In addition to changes in expression of genes involved in mitochondrial fission and fusion, this study also investigated changes in genes involved with ATP production. Ang II treatment caused no change in expression of ATP synthase, the protein involved directly in ATP synthesis (Figure 9A). In addition, it caused no significant change in expression of UCP3, a mitochondrial uncoupler (Figure 9E). Again, since the Ang II treatment was only for 3 hours, it is possible that the treatment must be longer to elicit any significant effects on these mitochondrial genes.

Ang II Effects on ROS

Ang II treatment induced significant increases in ROS production after 1 and 3 hours (Figure 10). Although there are significant increases in ROS production throughout the 3 hour time course, there is a drop at 2 hours. At this point, the cause of this biphasic change ROS production is not clear. Even though there were significant changes in ROS production at this time, there are not significant changes in oxidative capacity. However, there were changes in incomplete oxidation and a reduction in total β -oxidation. Many other studies have shown that ROS production, especially via NADPH oxidase, is increased with Ang II treatment, however more studies must be done to link this to mitochondrial dysfunction (Puri, Avantaggiati et al. 1995; Wei, Sowers et al. 2006; Cooper, Whaley-Connell et al. 2007; Diamond-Stanic and Henriksen 2010; Herrera, Silva et al. 2010; Phillips, Pechman et al. 2010).

Despite changes in ROS production, there were no changes in mRNA expression of catalase and SOD2, cellular antioxidants (Figure 11). In addition, there was no change in protein expression of SOD2 (Figure 12). However, there can be changes in ROS production without changes in cellular antioxidants, so this result is not necessarily surprising. However, this could mean that the cell is not increasing protection against ROS, even when there is more present, thus resulting in even more damage.

Benicar® Effects on Skeletal Muscle Oxidation and Enzyme Activity

Treatment of subjects with Benicar® resulted in no changes in fatty acid or glucose oxidation or in the activity of any enzymes. Several studies have shown that treatment with ARBs reduces new-onset diabetes and improves whole body glucose uptake and insulin sensitivity, however there are currently no studies that investigate the effects on oxidation (Scheen 2004; Sloniger, Saengsirisuwan et al. 2005; Henriksen 2007). The lack of an effect of Benicar® in the current study could be due to a number of factors. The first, and most obvious, is the limited N size. The second is the variability of subjects in both gender and BMI. The third is that many of the subjects did not exhibit many factors associated with the metabolic syndrome. Studies suggest that differences in Ang II treatment are only seen with obese subjects, who already have some metabolic dysfunction (Buchanan, Thawani et al. 1993; Townsend and DiPette 1993; Jonk, Houben et al. 2010). Perhaps studying only research participants that possessed traits associated with metabolic syndrome would have yielded more robust and less variable results.

Summary

Overall, the cell culture results show that Ang II affects substrate metabolism and mitochondrial oxidative capacity. The consistent finding was that Ang II treatment had to be present at the time of measuring substrate metabolism to observe any effects. Interestingly, the effects of Ang II when present in metabolism studies differed if cells were pretreated for 24 hours. Pretreatment for 24 hours seemed to elicit an overall reduction in oxidative capacity. However, the presence of Ang II during the 3 hour measure of substrate metabolism with no 24 pretreatment caused a reduction in fatty acid oxidation and an increase in glucose oxidation. The observed changes in substrate metabolism with the acute 3 hour treatment with Ang II occurred in concert with biphasic increases on ROS production. The cause and effect relationship between ROS production, decreased fatty acid oxidation, and increased glucose oxidation is not clear at this time.

The clinical study data showed no differences as a result of treatment with Benicar®, however this could be due to a small N size and the heterogenous group that was studied.

Future Directions

Recent studies suggest that the RAS is closely linked with the TLR4 pathway since many of the enzymatic and metabolic changes are the same (Eley, Russell et al. 2008). In addition, studies suggest that activation of NF- κ B is causing many of these metabolic changes because of the increase in inflammation, which is linked to a reduction in oxidative metabolism (Wei, Sowers et al. 2008; Frisard, McMillan et al. 2010). Therefore, it would be interesting to investigate this link more closely. Perhaps looking

at some inflammatory markers, such as interleukin 6 (IL-6) or components of the TLR4 or MAP kinase signaling pathway after Ang II treatment would provide some insight into how closely these pathways are linked and whether the metabolic effects as a result of Ang II are as a result of this linkage.

Additionally, the studies for RNA and protein as well as lipid synthesis were only performed after 3 hour treatments. Since changes in oxidation are seen with a longer term treatment, investigating the effects on gene and protein expression and neutral lipid synthesis after a 24 hour treatment could be beneficial. Additionally, since human studies suggest that some of the dysfunction associated with Ang II treatment is only seen with obese individuals, comparing metabolic effects of Ang II in muscle cell cultures from nonobese vs. obese humans may provide more insight into mechanistic action (Buchanan, Thawani et al. 1993; Townsend and DiPette 1993; Jonk, Houben et al. 2010).

In addition, it is curious why there is a reduction in ROS production at 2 hours, with a subsequent increase at 3 hours. The questions remains as to whether this ROS production is occurring via NADPH oxidase or the electron transport chain in the mitochondria. In cell lines other than skeletal muscle, it has been established that ROS production as a result of Ang II is mediated by NADPH oxidase (Doughan, Harrison et al. 2008; Daiber 2010). To investigate the source of ROS from these Ang II treatments in skeletal muscle cells, the ROS studies should be conducted using the NADPH oxidase antagonist, apocynin or with the electron transport chain inhibitor rotenone. When blocking NADPH oxidase, any ROS production seen can be attributed to mitochondrial sources. Conversely, any ROS production seen with rotenone treatment, blocking mitochondrial ROS sources, can be attributed to NADPH oxidase. Also, since all of the

ROS experiments were performed under acute (3 hour) Ang II treatment conditions, which is not when overall reduction in oxidative capacity was observed, investigating ROS production after 24 hours may be insightful.

Though no significant results were seen with oxidation or enzyme activity with the human study, more analysis is still yet to be conducted. Analysis of insulin signaling, protein and mRNA expression of certain genes, as well as histochemical analysis is still to be performed. However, as mentioned above, the low number and large variability of subjects is going to make it difficult to see any significant results. In addition, since studies suggest that effects may only be seen in already dysfunctional populations, such as those exhibiting symptoms associated with metabolic syndrome, it would be interesting to investigate the effects of ARBs on this population.

Chapter 6: References

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