EFFECTS OF ENHANCED EXTERNAL COUNTERPULSATION (EECP) ON GLYCEMIC CONTROL AND ARTERIAL FUNCTION IN PATIENTS WITH ABNORMAL GLUCOSE TOLERANCE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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This dissertation is dedicated to my wife, Allison Marie Martin, and my family. Their love and support have carried me through this very rewarding chapter of my life. I am truly blessed to have so many wonderful people in my corner. I would not be who I am today without them. They provide me with the confidence to succeed in wherever the road ahead may take us.
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<tr>
<td>4-HNE</td>
<td>4-hydroxynoneal</td>
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<tr>
<td>6-keto-PGF-1α</td>
<td>6-keto-prostaglandin 1α</td>
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<tr>
<td>8-iso-PGF2α</td>
<td>8-iso-prostaglandin 2α</td>
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<tr>
<td>AAPH</td>
<td>2,2’-Azobis-2-methyl-propanimidamide, dihydrochloride</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
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<tr>
<td>AGEs</td>
<td>advanced glycation endproducts</td>
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<tr>
<td>AGT</td>
<td>abnormal glucose tolerance</td>
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<tr>
<td>AICAR</td>
<td>aminomimidazole carboxamide ribonucleotide</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>AMPK</td>
<td>5’-adenosine monophosphate-activated protein kinase</td>
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<td>ARB</td>
<td>angiotensin-II receptor blocker</td>
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<td>AS160</td>
<td>Akt substrate of 160 kDa</td>
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<tr>
<td>ATF-2</td>
<td>activating transcription factor-2</td>
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<td>ATP</td>
<td>adenosine tri-phosphate</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BH₄</td>
<td>tetrahydrabiopterin</td>
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<td>CAD</td>
<td>coronary artery disease</td>
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<td>CAMKII</td>
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<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
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<td>C-reactive protein</td>
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<td>DDAH</td>
<td>dimethylarginine dimethlaminoedrolase</td>
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<td>dual energy x-ray absorptiometry</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EDV</td>
<td>endothelium dependent vasodilatation</td>
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<td>enhanced external counterpulsation</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>flow mediated dilation</td>
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<td>H₂O₂</td>
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<td>HGF</td>
<td>hepatocyte growth factor</td>
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<td>high sensitivity C-reactive protein</td>
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<td>IFG</td>
<td>impaired fasting glucose</td>
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<td>impaired glucose tolerance</td>
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<td>c-Jun-NH&lt;sub&gt;2&lt;/sub&gt; terminal kinase</td>
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<td>oral glucose tolerance testing</td>
</tr>
<tr>
<td>ORAC</td>
<td>oxygen radical absorbance capacity</td>
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<tr>
<td>P160myb</td>
<td>myb-binding protein 1A</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide 3-kinase dependent kinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>peroxisome proliferator-activated receptor-γ coactivator 1α</td>
</tr>
<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>prostacyclin</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>PIP2</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
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<tr>
<td>PIP3</td>
<td>phosphatidylinositol (3,4,5)-triphosphate</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>cyclic GMP-dependent kinase/protein kinase G</td>
</tr>
<tr>
<td>PPG&lt;sub&gt;120&lt;/sub&gt;</td>
<td>post-prandial glucose at 120 minutes</td>
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<tr>
<td>PRMTs</td>
<td>protein-arginine-N-methyltransferase family of enzymes</td>
</tr>
<tr>
<td>QUICKI</td>
<td>quantitative insulin sensitivity check index</td>
</tr>
<tr>
<td>RabGAP</td>
<td>RabGTPase activating protein</td>
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<tr>
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<td>succinate dehydrogenase</td>
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<tr>
<td>SNAP</td>
<td>S-nitroso-N-penicillamine</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>sVCAM</td>
<td>soluble vascular cell adhesion molecule</td>
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<tr>
<td>T2DM</td>
<td>Type II Diabetes Mellitus</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β activated kinase 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>TBC1D1</td>
<td>TBC1 domain family member 1</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
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<tr>
<td>TMB</td>
<td>tetramethylbenzadine</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<td>thiazolidinedione</td>
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<td>vascular endothelial growth factor</td>
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Enhanced external counterpulsation (EECP) is a noninvasive modality used to treat patients with refractory angina. The salutary benefits of EECP treatment include increases in peripheral vascular function and nitric oxide bioavailability. Nitric oxide (NO) is also known to upregulate the translocation and expression of the major glucose transporter in muscle, glucose transporter 4 (GLUT-4). Of critical importance, is the fact that this NO signaling pathway is distinct from and/or additive to insulin-stimulated skeletal muscle glucose uptake, which is notoriously impaired in humans with abnormal glucose tolerance (AGT). Moreover, the effects of EECP on skeletal muscle morphology and glucose uptake signaling are largely unknown. Therefore, the purpose of this study was to investigate the effects of EECP treatment on glycemic control and arterial function in patients with AGT.

Eighteen (n = 18) patients with AGT were recruited for the study. They were randomly assigned to receive either 35 1-hour sessions of EECP (n = 12) or 7-weeks of standard care (n = 6). Peripheral vascular function, vasoactive balance, oxidative stress, inflammation, skeletal muscle morphology, skeletal muscle protein expression,
fasting glycemic control, and dynamic glucose tolerance were measured before and after 35 1-hour sessions of EECP or Time-Control.

EECP resulted in an increase in normalized brachial artery (27%) and popliteal artery (52%) flow-mediated dilation as well as peak (26%) and total (37%) forearm hyperemic blood flow. Plasma nitrite/nitrate (NOx) increased (30%) and asymmetric dimethylarginine (ADMA) decreased (11%) following 35 sessions of EECP. Additionally, 8-isoprostane-PGF-F2α (8-iso-PGF2α), a marker of lipid peroxidation in the plasma, decreased (23%) with a concurrent decrease (28%) in high sensitivity C-reactive protein (hsCRP), a marker of inflammation. Analysis of vastus lateralis skeletal muscle biopsies revealed significant increases in endothelial nitric oxide synthase (eNOS) (87%) and GLUT-4 protein expression (47%) following EECP. Furthermore, there was a significant elevation in humoral concentrations of vascular endothelial growth factor (VEGF) (75%) and the capillary-to-fiber ratio (8%) following EECP. Fasting plasma glucose (FPG) was reduced by 16.9 mg/dL and the homeostasis model assessment of insulin resistance (HOMA\textsubscript{IR}) decreased (31%) following EECP. Moreover, plasma glucose concentrations 120 minutes after initiation of an oral glucose test (OGTT) decreased (224.4 mg/dL vs. 196.1mg/dL) and the whole-body composite insulin sensitivity index (ISI composite) increased (21%).

The improvements in NO bioavailability and endothelial function observed in the present study, as well as capillary to fiber ratios, likely mediate greater delivery of nutrients to ‘nutritive’ tissues (i.e. skeletal muscle) at rest and during glycemic challenge. Additionally, NO signaling been has implicated in regulation of GLUT-4 translocation and glucose uptake. The multifaceted nature of vascular function and
glycemic control makes it difficult to isolate a single mechanism responsible for these adaptations. However, novel evidence from the present study supports the hypothesis that decreasing nitric oxide bioavailability contributes to the pathology of AGT. Interventions that improve NO bioavailability may be attractive treatment strategies for patients with AGT.
CHAPTER 1
INTRODUCTION

Background

An estimated 23.6 million people in the United States suffer from Diabetes Mellitus (DM).\textsuperscript{1} Of the estimated 23.6 million with DM, Type II DM (T2DM) accounts for 90-95% of all diagnosed cases.\textsuperscript{1} After the age of 60, the prevalence of T2DM climbs to 23.1% of all people. The prevalence of DM has more than doubled since 1990 leading to the designation of DM as an epidemic in the United States. T2DM is often associated with the co-morbidities; obesity, hypertension, and hyperlipidemia. The combination of these risk factors is known as metabolic syndrome. Cardiovascular disease is the leading cause of morbidity and mortality in patients with T2DM, with heart disease and stroke noted as cause of death on 84% of death certificates.\textsuperscript{1, 2} The sequela of vascular disease extends to amputations, blindness, myocardial infarction, and stroke. Chronic exposure to hyperglycemia is the primary cause of cardiovascular disease in patients with T2DM and the risk of microvascular complications is considerably reduced by better glycemic control.

Another 56 million Americans are estimated to be pre-diabetic, and among people aged 20 years or more in America a staggering 37% of the population is classified as pre-diabetic or having T2DM.\textsuperscript{1} Furthermore, aging is a significant risk factor for the development of pre-diabetes. Indeed, among American adults aged 40-74 years, 40.1% had prediabetes.\textsuperscript{1} Pre-diabetes is a condition in which blood glucose levels are higher than normal, but not high enough to be classified as T2DM. Pre-diabetes markedly increases the risk for progression to T2DM as well as cardiovascular disease and stroke.\textsuperscript{3-5}
Impaired glucose tolerance (IGT) is defined as a plasma glucose concentration of
110 to 125 mg/dL after overnight fast and a plasma glucose concentration of 140 to 199
mg/dL after a 2-hour oral glucose tolerance test (OGTT).\(^6\) Individuals with plasma
glucose concentrations that are greater than the highest range of IGT for fasting and 2-
hour OGTT plasma glucose concentrations are classified as having T2DM. The term
abnormal glucose tolerance (AGT) includes individuals with IGT and T2DM. T2DM,
previously referred to as non-insulin dependent or adult onset diabetes, and AGT is
characterized by the marked decline in insulin mediated glucose transport in skeletal
muscle and adipose tissue. This phenomenon, by which the cells do not respond
appropriately to endogenous circulating insulin, is referred to as insulin resistance. A
consequence of insulin resistance is the inability to clear circulating plasma glucose
which is reflected by the abnormally elevated fasting and/or post-prandial plasma
glucose concentrations. Because skeletal muscle accounts for 65-90% of the clearance
of a hyperglycemic challenge,\(^2,7,8\) interventions that increase skeletal muscle glucose
uptake are known to improve glucose homeostasis in patients with type T2DM.\(^8-10\)

Treatment and management of T2DM can range from alteration of diet and
physical activity habits to pharmacotherapy. However, complications often associated
with DM, such as neuropathy and obesity, can often limit the ability of persons to
increase their physical activity in an effort to manage their condition. Additionally, a
lifetime of refusal to be physically active may be an indication that there is little
willingness to change physical activity habits. Physical activity can help with the
management of glycemic control in a variety of ways, but in situations where it may not
be possible or pertinent, enhanced external counterpulsation (EECP) may be an effective means by which to increase insulin sensitivity and/or glucose metabolism.

**Antihyperglycemic Treatment Options**

Current treatment strategies to achieve adequate glycemic control in patients with AGT include exercise training and anti-hyperglycemic drug regimens. However, compliance with home-based exercise guidelines in patients with AGT is abysmally low. Indeed, data from two very recent population based surveys (a total of 345 patients) revealed that only 25% of patients with T2DM participate in exercise a minimum of 1 hour per week, despite being under the care of physicians who recommended exercise as treatment. This finding is consistent with earlier studies reporting very low compliance with exercise recommendations among patients with T2DM. Relevant also, is the fact that 60-70% of diabetic patients have mild to severe nervous system damage. Neuropathy in either upper or lower body extremities can be a contraindication for participation in exercise therapy for patients with diabetes.

Patient compliance with drug therapy for glycemic control is only slightly better than exercise compliance. Daily et al. studied a very large cohort consisting of 37,431 patients with T2DM to determine compliance with anti-hyperglycemic drug regimens. At the 1-year follow-up, only 49% of patients receiving monotherapy and just 36% of patients receiving polytherapy were compliant with anti-hyperglycemic drug regimens. At the 2-year follow-up, compliance deteriorated even further and only 42% of patients receiving monotherapy and 29% of patients receiving polytherapy remained compliant with their drug regimens. Considering the results of exercise and drug compliance studies, it is clear that a high percentage of patients with AGT do not achieve adequate
glycemic control. Consequently, alternative methods that improve glucose homeostasis should be considered.

In this proof-of-concept study design, we explored an alternative anti-hyperglycemic therapy for patients with AGT that is feasible, noninvasive, exercise-independent and perhaps more cost-effective than metabolism enhancing medications. The goal of this application was to determine the efficacy and underlying mechanisms responsible for improving glycemic control in patients with AGT following 35 1-hr sessions of EECP.

**Glucose Uptake Pathways in Skeletal Muscle**

Glucose uptake in skeletal muscle cells is regulated by 3 known pathways: 1) insulin-mediated cell signaling; 2) contraction-mediated cell signaling; 3) Nitric Oxide (NO)-mediated cell signaling. A brief outline of each pathway and the potential relevance of EECP as a modulator of Pathway #3 are presented below.

**Insulin mediated glucose uptake (Pathway #1)**

The insulin mediated pathway of glucose uptake is dependent upon phosphoinositide 3-kinase (PI3K) activation via receptor associated tyrosine kinase activity and ultimately glucose transporter 4 (GLUT-4) translocation to the plasma membrane. PI3K activation recruits both protein kinase B (Akt) and phosphoinositide 3-kinase dependent kinase (PDK1) to the cell membrane where PDK1 phosphorylates and activates Akt. Subsequently, Akt phosphorylates Akt substrate of 160 kDa (AS160) at its RabGTPase activating domain (RabGAP) which inhibits its activity and removes inhibition of Rab protein activity. As a consequence of greater Rab protein activity, GLUT-4 translocation to the cell membrane is realized. This pathway is notoriously impaired in humans with AGT.\(^1^7\),\(^1^8\)
Contraction mediated glucose uptake (Pathway #2)

The insulin/PI3K independent pathway, commonly referred to as the contraction/hypoxia pathway, mediates glucose uptake in skeletal muscle due to perturbations in the vasculature and skeletal muscle when compared to the basal state. One mechanism by which contraction mediated glucose uptake is realized is through 5′-adenosine monophosphate-activated kinase (AMPK) activity. An increase in the adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio within the cell (e.g. as during contractions) is known to be an activator of AMPK activity, and correlates strongly with glucose uptake.19 AMPK phosphorylates AS160 relieving Rab protein inhibition and resulting in GLUT-4 translocation. AMPK activity also increases expression of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), a master regulator of cellular metabolism, which leads to an increase in GLUT-4 protein transcription and translation.19 Therefore, AMPK signaling mediates acute changes in GLUT-4 translocation from existing pools and a later increase in GLUT-4 protein amount. The insulin and contraction-mediated glucose uptake pathways are additive, supporting the idea that they are two distinct pathways. However, a point of convergence in the two pathways appears to occur at AS160 phosphorylation. This pathway is the therapeutic target of exercise in patients with AGT.

Nitric oxide (NO) mediated glucose uptake (Pathway #3)

A third signaling pathway uses NO to stimulate glucose transport in muscle. NO increases glucose uptake through a mechanism that is distinct from the insulin-mediated (Pathway #1) and contraction-mediated (Pathway #2) pathways in muscle. NO increases GLUT-4 protein expression and GLUT-4 translocation to the cell membrane via a cyclic guanosine monophosphate (cGMP) and AMPK-dependent
It is well established that muscle tissue expresses significant NO production at rest from both endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS). However, eNOS is typically found in highest concentrations around the mitochondria and is thought to play a greater role in cell glucose metabolism. Indeed, in eNOS knockout mice both basal and insulin-stimulated glucose uptake in skeletal muscle are reduced by 40%, when compared to wild-type controls. Critically relevant to this proposal, is the fact that patients with AGT are more reliant upon the NO pathway for glucose uptake than age-matched euglycemic controls. Kingwell et al. found that nitric oxide synthase (NOS) inhibition with $\text{N}^\text{G}$-monomethyl-L-arginine (L-NMMA) reduced leg glucose uptake by 75% in patients with T2DM, but only by 34% in non-diabetic control subjects.

In contrast, sodium nitroprusside (SNP), a NO donor, significantly increases glucose uptake in a dose-dependent manner and the responsible mechanism appears to be translocation of GLUT-4 to the cell membrane. NO donors significantly increase the activity of AMPK by acting on the $\alpha_1$ catalytic subunit and AMPK can act in positive feedback loop with NOS further augmenting NO production and AMPK activity. The increase in AMPK activity could explain the increase in NO mediated glucose uptake due to increasing GLUT-4 translocation and PGC-1α transcription. Lira et al. has previously shown that the NO donor S-nitroso-$N$-penicillamine (SNAP) increased GLUT-4 protein expression by 65% in L6 myotubes via a cGMP and AMPK-dependent mechanism. Moreover, L6 myotubes exposed to aminoimidazole carboxamide ribonucleotide (AICAR), an AMPK agonist, experienced a nine-fold increase in GLUT-4 mRNA. In vivo, GLUT-4 messenger ribonucleic acid (mRNA) was increased nearly
two-fold in the rat plantaris muscle 12 hrs after AICAR injection.\textsuperscript{5} Thus, evidence from this assisting laboratory corroborates that in skeletal muscle, NO concentration likely influences GLUT-4 expression via control of cellular activity of AMPK kinases.

Lastly, NO donors significantly increase skeletal muscle glucose uptake in the presence of wortmannin, a PI3K inhibitor, indicating that the NO signaling pathway is not a PI3K dependent mechanism (i.e. Pathway #1 above).\textsuperscript{20} Clearly, the contraction and NO mediated glucose uptake pathways overlap downstream of AMPK. However, L-NG-nitroarginine methyl ester (L-NAME), another NOS inhibitor, does not abolish contraction mediated glucose uptake in skeletal muscle (i.e. Pathway #2 above).\textsuperscript{20} Thus, there is excellent evidence indicating that the NO mediated glucose uptake pathway in muscle is independent of both the insulin and contraction mediated mechanisms, making NO bioavailability an attractive target for circumventing impaired insulin sensitivity and augmenting exercise effects. In summary, increasing the skeletal muscle NO concentration has an antiglycemic effect, whereas blocking the role of NO results in marked hyperglycemia and insulin resistance.

**Principles of Enhanced External Counterpulsation (EECP)**

Intermittent external compression of skeletal muscle is known to upregulate eNOS protein and NO bioavailability in animal studies.\textsuperscript{25, 26} Our lab (Braith et al.),\textsuperscript{27} and others,\textsuperscript{28, 29} have demonstrated that external compression upregulates NO bioavailability in humans. We hypothesized that external compression of the large muscle groups of the lower body using EECP would increase muscle perfusion, increase intramuscular eNOS and NO expression, increase GLUT-4 protein, and chronically improve glycemic control in patients with AGT. EECP is an established, Food and Drug Administration and Medicare-approved, non-invasive therapy for patients with coronary artery disease.
(CAD) who experience persistent refractory angina, despite standard revascularization procedures and/or aggressive anti-angina medication. EECP has proven to be especially efficacious in patients with CAD who are not amenable to percutaneous coronary intervention or bypass surgery, because of unsuitable coronary anatomy, multiple previous revascularization attempts, age, additional co-morbid conditions, or patient preference. Data from most clinical trials and the International Patient Registry demonstrate that EECP is effective in reducing anginal symptoms and nitroglycerin usage, increasing exercise tolerance, and decreasing the need for hospitalization. The clinical benefits of EECP have been sustained for 2, 3, and 5 years after treatment in most patients.

EECP utilizes equipment to inflate and deflate 3 compressive cuffs enclosing the lower extremities. During an EECP treatment session, the patient lies on a padded table in which sets of electronically controlled inflation and deflation valves are located. These valves are connected to the 3 pneumatic cuffs, wrapped around the calves, lower thighs, upper thighs and buttocks. The cuffs are sequentially inflated to approximately 300 mmHg with compressed air from distal to proximal in early diastole and rapidly deflated at the onset of systole. Inflation and deflation of the cuffs are triggered by events in the cardiac cycle via microprocessor-interpreted ECG signals. A standard course of EECP treatment consists of 35 1-hour sessions distributed over a 7-week period.

Central angiogenesis mechanism of EECP

The central hypothesis, in most investigations conducted to elucidate the mechanism of action, was that EECP may promote coronary angiogenesis through robust diastolic pressure augmentation during rapid cuff inflation, analogous to intra-
aortic balloon counterpulsation. However, this understanding of EECP is only a theory and remains unconfirmed in clinical trials.\textsuperscript{28, 35} In an international trial (7 Centers; 175 chronic stable angina patients), EECP failed to elicit improved cardiac perfusion in 46% of study subjects.\textsuperscript{42} More recently, a multicenter trial involving 6 United States University Hospitals showed that EECP failed to improve myocardial blood flow to and within ischemic regions of the myocardium either at rest or during exercise stress in 37 patients with CAD, as assessed by quantitative gated single photon emission computed tomography.\textsuperscript{43} However, despite negligible improvements in myocardial perfusion, approximately 85% of patients in EECP clinical trials experience reduction in angina.\textsuperscript{31, 42} Consequently, Dr. Braith and coworkers reasoned that the traditional “coronary angiogenesis” understanding of EECP required extensive ‘revision’ and was inadequate to explain the efficacy of therapy (i.e. angina reductions) in patients with CAD. They found that peripheral endothelial function was the primary therapeutic target for EECP.\textsuperscript{27}

**Peripheral vascular mechanism of EECP**

Our laboratory recently completed the first randomized, sham-controlled study to investigate the extra-cardiac effects of EECP on endothelial function in CAD patients.\textsuperscript{27} In that study, the primary hypothesis was that improvement in peripheral arterial stiffness and endothelial function are the mechanisms underlying the chronic anti-ischemic clinical benefits of EECP by reducing myocardial oxygen demand. To test this hypothesis, 42 symptomatic patients with CAD were randomized (2:1 ratio) to either 35 1-hour sessions of EECP (n=28) or Sham EECP (n=14). In brief, overwhelming biochemical and functional evidence that EECP elicits systemic improvements in vascular biology was found. EECP increased plasma levels of NO by 36% and decreased endothelin-1 (ET-1) by 25%. NO bioavailability was enabled through the
anti-inflammatory effects of EECP, as evidenced by significant reductions in the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α: - 16%), C-reactive protein (CRP: - 9%), monocyte chemotactic protein-1 (MCP-1: - 13%), and soluble vascular cell adhesion molecules (sVCAM: - 6%). NO bioavailability was further enabled through improvement in systemic redox balance, as evidenced by a 21% decrease in plasma levels of 8-iso-prostaglandin F2α (8-iso-PGF2α), which is viewed as the most valid plasma marker to assess systemic oxidative stress. Endothelial-dependent flow mediated dilation (FMD) in the brachial and femoral arteries was increased by 51% and 30%, respectively and peak blood flow in the forearm and calf was increased by 18% and 16%, respectively. Improved endothelial function and reduced afterload following EECP treatment resulted in decreased angina symptoms. Patients reduced their Canadian Cardiovascular Society angina classification from 3.1 to 1.2 (mean ± SEM; 4 point scale), daily angina episodes by 72%, and daily nitrate usage by 81%.

**EECP and skeletal muscle glucose metabolism**

The primary mechanism responsible for upregulation of eNOS and NO following EECP therapy is related to the intermittent bouts of hemodynamic shear stress created with each inflation-deflation cycle of the 3 pneumatic cuffs. Shear stress is a primary stimulus for the synthesis and release of endothelial-derived NO. The blood flow shear stress stimulus is transduced into the endothelial cell via the integrin/cytoskeleton mechano-transduction pathway. It is well documented that sequential inflation (~ 300 mm Hg) of the 3 pneumatic EECP cuffs from calf to buttocks during diastole produces a robust retrograde pressure wave in the central aorta that subsequently increases coronary artery perfusion pressure, analogous to intraaortic balloon counterpulsation. However, inflation of the EECP cuffs also produces high-pressure retrograde blood flow.
in the femoral arteries and simultaneous moderate-pressure antegrade flow in the brachial arteries.\textsuperscript{26, 46} Using color Doppler imaging in a porcine EECP model, Zhang and coworkers\textsuperscript{26} found that brachial artery blood flow velocity increased by 132\% (59 versus 24 cm/second; \( p < 0.001 \)) and brachial artery wall shear stress increased by > 200\% (49 versus 23 dyne/cm\(^2\); \( p < 0.001 \)) during lower body compression of EECP pneumatic cuffs. Recent evidence suggests that arterial exposure to this type of pulsatile, hemodynamic shear stress, compared to steady laminar flow, may be the most critical factor affecting endothelial function.\textsuperscript{47} Data from a randomized, sham-controlled study suggest that the significant increases in pulsatile and oscillatory flow during EECP treatment, as recorded by color Doppler, may provide a form of “massage” on the endothelium and improve its function.\textsuperscript{27} That is, each session of EECP may be thought of as providing a direct dose of vascular medicine.

Support for our EECP hypothesis can be found in an animal model of external compression. Tan et al.\textsuperscript{25} applied intermittent pneumatic compression (55 mmHg pressure achieved in <1 second) to the hind limbs of 16 male Sprague-Dawley rats for the durations of 0.5, 1.0, and 5 hours. Following intermittent compression, eNOS in skeletal muscle homogenate was upregulated to 120\%, 180\%, and 270\% from baseline, respectively. Similarly, nNOS expression was up-regulated, but to a lesser degree. Furthermore, EECP increased plasma levels of NO by 36\% in CAD patients following 35 1-hour sessions in a sham controlled study.\textsuperscript{27} Indices of arterial and endothelial function were equally impressive after 35 1-hour sessions of EECP as endothelial-dependent flow mediated dilation (FMD), a non-invasive measure of NO bioavailability, in the brachial and femoral arteries was increased by 51\% and 30\%, respectively.\textsuperscript{27}
These results, from this animal model resembling EECP, and from data in our own laboratory, serve to confirm our hypothesis that compression-induced improvement in vascular function is mediated by modulation of NO bioavailability and by regulating expression of NOS isoforms, in particular eNOS.

While it has been well established that EECP causes an acute increase in blood flow to the muscle tissue, the effects of EECP on skeletal muscle signaling and metabolism have not been well characterized. A previous study by Crenshaw et al. has shown that application of external pressure to human cadaver limbs there is a linear relationship between external and intramuscular pressures (1988). Furthermore, in a rodent model of intermittent pneumatic compression (IPC), pressure underneath the cuffs closely resembles the pressure programmed on the compression unit. L6 myotubes that have been pressurized to mimic intramuscular pressures during walking or running showed a significant increase in succinate dehydrogenase (SDH) activity and glucose uptake with a decrease in lactate release. Furthermore, a single 30 minute bout of EECP therapy in humans has been shown to significantly decrease plasma glucose concentration. Although the mechanism by which external pressure augments metabolism has yet to be elucidated, available evidence suggests that mechanical pressure, devoid of active contraction, can increase metabolism and potentially alter AMP/ATP ratios. Indeed, in an acute rodent model of intermittent pneumatic compression, the arterial-venous oxygen difference was shown to increase during compressions. This is indicative of oxygen consumption and supports an increase in tissue metabolism during external compression. Furthermore, a studies by Grayson et al., demonstrated a significant increase in somatic oxygen consumption in
humans during 30 minutes of EECP therapy. Another potential mechanism for the perturbation in skeletal muscle metabolism during IPC is stretch of the muscle tissue directly under and in the immediate vicinity of cuff edges. Indeed, NO production increases approximately 20% in isolated soleus muscle subjected to passive stretch. Furthermore, myotubes subjected to cyclic stretching for 2 hours showed a 42% increase in NOS activity from basal rates. Perhaps of long-term consequence of EECP compression, mechanical loading may upregulate nNOS expression as a result of membrane associated proteines responsive to mechanical disruption. Mechanical loading has been shown to regulate expression of talin in C2C12 myotubes and nNOS protein expression is slightly elevated following IPC in underlying tissue following 1 hour of treatment.

Specific Aims and Hypotheses

**Specific aim 1:** Determine whether 35 1-hour sessions of EECP therapy will elicit changes in patient fasting glycemic control, HbA1c levels, and insulin sensitivity as measured by oral glucose tolerance testing (OGTT) in subjects with AGT.

**Hypothesis 1:** 35 1-hour sessions of EECP in subjects with AGT will decrease fasting plasma glucose concentrations (relative to plasma insulin concentrations), glycosylated hemoglobin (HbA1c) levels, and indices of insulin sensitivity as determined by oral glucose tolerance testing (OGTT) compared to time matched controls.

**Specific aim 2:** Determine whether EECP and accompanying changes in skeletal muscle NO bioavailability will induce upregulation of the AMPK-PGC-1α cell signaling pathway and ultimately increase GLUT-4 expression when assessed in muscle biopsy samples.
Hypothesis 2: 35 1-hour sessions of EECP in subjects with AGT will upregulate the AMPK-PGC-1α cell signaling pathway as assessed by PGC-1α, GLUT-4, AMPK, AS160, and Akt protein concentrations and phosphorylation state in western blots of vastus lateralis biopsy homogenate compared to time matched controls.

Specific aim 3: Determine whether EECP, as compared to standard care, improves endothelial function in peripheral muscular arteries (brachial and popliteal) and resistance arterioles (forearm and calf) in patients with AGT after 35 1-hour sessions.

Hypothesis 3: 35 1-hour sessions of EECP in subjects with AGT will increase endothelial function in the peripheral muscular arteries and resistance arterioles, as determined by flow mediated dilation (brachial and popliteal) and venous occlusion plethysmography (forearm and calf) respectively, compared to time controls.

Specific aim 4: Determine whether changes in endothelial function are accompanied by commensurate changes in humoral levels of vasoactive agents including nitric oxide (nitrate/nitrite), prostacyclin (6-keto-PGF-1α), and ET-1.

Hypothesis 4: 35 1-hour sessions of EECP in subjects with AGT will have beneficial effects on the plasma levels of vasoactive agents compared to time matched controls.

Specific aim 5: Determine whether systemic vascular changes are accompanied by commensurate changes in skeletal muscle capillarity, eNOS and nNOS protein concentration, and humoral concentration of vascular endothelial growth factor (VEGF) when assessed in biopsy samples taken from the vastus lateralis and blood draws.
Hypothesis 5: 35 1-hour sessions of EECP in subjects with AGT will increase eNOS and nNOS protein concentration measured in skeletal muscle biopsy homogenate, capillary density in serial sections of skeletal muscle biopsy samples, and humoral concentration of VEGF compared to time matched controls.
CHAPTER 2
LITERATURE REVIEW

Abnormal Glucose Tolerance (AGT)

A hallmark of insulin resistance, a disorder in which the cells do not use insulin properly, is abnormal glucose tolerance (AGT). Abnormal glucose tolerance includes individuals with impaired glucose tolerance (IGT) and Type II Diabetes Mellitus (T2DM). Individuals with IGT, but not T2DM, are classified as pre-diabetics. Pre-diabetes is a condition in which blood glucose levels are higher than normal, but not high enough to be classified as diabetes. Pre-diabetes markedly increases the risk for progression to T2DM as well as cardiovascular disease and stroke. The 2006 World Health Organization (WHO) recommendation for diagnosis of pre-diabetes is the presence of either impaired fasting glucose (IFG) and/or IGT.\(^6\) IFG is defined as a plasma glucose concentration of 110 to 125 mg/dL after overnight fast. IGT is defined as a plasma glucose concentration of 110 to 125 mg/dL after overnight fast and a plasma glucose concentration of 140 to 199 mg/dL after a 2-hour OGTT.\(^6\) According to the most recent data from the Centers for Disease Control (CDC), amongst adults aged 40-74 years in the United States, 33.8% had IFG, 15.4% had IGT, and 40.1% had pre-diabetes.\(^1\) The WHO estimates that about 10% of people worldwide have IGT.\(^6,54\) In addition, the CDC estimate of the prevalence of T2DM in the United States was 23.6 million people, or 7.8% percent of the population.\(^1\) T2DM is defined as a fasting plasma glucose concentration of >125 mg/dL or a plasma glucose concentration of > 200 mg/dL after a 2-hour oral glucose tolerance test (OGTT).\(^6\) The total (direct and indirect) costs of diabetes in the United States for 2007 was estimated to be 174 billion dollars.\(^1\) The WHO projects the prevalence of diabetes to more than double in the next 30 years,
placing a significant burden on not only the population as a whole, but also health care costs.\textsuperscript{6, 54}

**Measures of Glycemic Control**

AGT, defined here as IGT or T2DM, is associated with insulin resistance as well as cardiovascular morbidity and all cause mortality.\textsuperscript{55} Insulin resistance is typically defined as decreased sensitivity to metabolic actions of insulin, such as insulin-mediated glucose disposal and inhibition of hepatic glucose production.\textsuperscript{56} Insulin has concentration dependent saturable actions to increase whole body glucose disposal, and several surrogates of insulin sensitivity are available for evaluation of glycemic control in humans. The gold standard, and most direct measure of insulin sensitivity, is the hyperinsulinemic euglycemic clamp (HEC). With this technique, glucose disposal in skeletal muscle and adipose tissue is maximized while hepatic glucose production is suppressed. Although the glucose infusion rate determined via HEC provides the most direct measure of peripheral insulin sensitivity, one still has to assume that hepatic glucose production is completely abolished.\textsuperscript{2} In addition, clamps are high risk, invasive procedures that are not normally used in clinical practice. The OGTT has been used to diagnose IGT and T2DM in clinical practice for many years. A major criticism of the OGTT is that the gastrointestinal influence on glucose disposal cannot be ignored. However, the OGTT may be the most clinically and physiologically relevant as oral ingestion of food stuffs is the primary means of nourishment. Several surrogates of insulin sensitivity have been derived from the OGTT that correlate very strongly with HEC testing.

Simple measures of fasting plasma glucose and insulin concentrations can provide an estimate of insulin resistance. Indices of insulin sensitivity from these measures
include the quantitative insulin sensitivity check index (QUICKI) and the homeostasis model assessment of insulin resistance (HOMA$_{IR}$). The HOMA$_{IR}$ correlates well with the glucose disposal rate derived from HEC$^{57, 58}$ although the correlation appears to weaken with higher levels of glycemia.$^{59}$ Among the criticisms of the HOMA$_{IR}$ are a large coefficient of variation and the pulsatile nature of insulin secretion. However, 3 separate samples, 5 minutes apart, of plasma insulin concentration were measured to determine a true average fasting plasma insulin concentration. The mathematical difference between the QUICKI and the HOMA$_{IR}$ is simply that the former uses the reciprocal of the logarithm of both glucose and insulin to account for the skewed distribution of fasting insulin values. Both indices correlate well with the HEC,$^{60}$ but some argue that the QUICKI may be applied to wider ranges of insulin sensitivity.$^{61, 62}$

Stronger surrogates of insulin sensitivity can be derived from the multiple sampling of plasma insulin and glucose during an OGTT. During an OGTT fasting blood samples are taken prior to the ingestion of a beverage containing 75 grams of glucose (‘sugar water’). Four additional samples of plasma insulin and glucose are then taken at 30, 60, 90, and 120 minutes after ingestion of the beverage. There are a plethora of indices based on the OGTT, but to highlight a few, the Matsuda,$^{58}$ Belfiore,$^{63}$ Stumvoll,$^{64}$ and Mari,$^{65}$ indices have all been shown to correlate significantly with the HEC with r values of 0.73, 0.96, 0.80, and 0.73 respectively.$^{59}$ Each index uses data from the entire OGTT, including area under the glucose and insulin curves, to determine insulin sensitivity. Importantly, these indices have been validated in a wide variety of lean and obese subjects with IGT, T2DM and/or normal glucose tolerance. An inherent limitation to indices of insulin sensitivity derived from the OGTT is that it is impossible to
differentiate between whole-body, peripheral, or hepatic insulin sensitivity. However, since this method of glucose ingestion is the most physiologically relevant, improvement in indices of insulin sensitivity from the OGTT are encouraging signs of improved glucose tolerance in subjects with AGT.

Finally, glycosylated hemoglobin (HbA1c) provides a long term index of glycemic control. Since the half life of a red blood cell is about 120 days, a single determination of HbA1c reflects the average blood glucose control level during the preceding 8 to 12 weeks. Therefore, HbA1c is used primarily to identify the average plasma glucose concentration over prolonged periods of time (2 to 3 months). It is formed in a non-enzymatic glycation pathway by hemoglobin’s exposure to plasma glucose. In diabetics, higher HbA1c values, indicating worse glycemic control, have been associated with cardiovascular disease nephropathy, and retinopathy. HbA1c is reported as percentage, and the equation used to determine estimated average glucose is the following: estimated average glucose (mg/dL) = 28.7 x HbA1c (%) – 46.7. Therefore, a small change in HbA1c values could indicate a large improvement in long term glycemic control. For example, exercise intervention has been shown to decrease HbA1c percentage by more than one half a percent indicating at least a 15 mg/dL change in average plasma glucose concentration.

**Insulin Mediated Glucose Uptake**

Insulin binding to its specific receptor promotes glucose transporter-4 (GLUT-4) trafficking to the plasma membrane. The primary, and well established pathway in the literature, is the phosphoinositide 3-kinase (PI3K) pathway. Insulin binding to the specific insulin receptor causes the insulin receptor substrate (IRS1/2) to recruit PI3K to the plasma membrane via receptor associated tyrosine kinase activity. Subsequent
PI3K activation catalyses the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) which recruits both protein kinase B (Akt) and phosphoinositide 3-kinase dependent kinase (PDK1) to the cell membrane where PDK1 phosphorylates and activates Akt. Activated Akt then phosphorylates Akt substrate of 160 kDa (AS160) at its RabGTPase activating protein (RabGAP) domain which inhibits its activity and removes inhibition of Rab protein activity. As a consequence of greater Rab protein activity, GLUT-4 translocation to the cell membrane is realized. Therefore, AS160 functions as a negative regulator of GLUT-4 trafficking under non-stimulated conditions where the active GAP domain of AS160 suppresses the activity of Rab proteins that are required for GLUT-4 translocation to occur. In individuals with AGT/insulin resistance, this glucose uptake pathway is notoriously impaired at one or several levels of the insulin signaling cascade in skeletal muscle.\textsuperscript{17, 18}

**Contraction Mediated Glucose Uptake**

The insulin/PI3K independent pathway, commonly referred to as the contraction/hypoxia pathway, mediates glucose uptake in skeletal muscle due to perturbations in the vasculature and skeletal muscle compared to the basal state. Two of the major players in contraction stimulated glucose uptake include 5'-adenosine monophosphate-activated protein kinase (AMPK) and calcium/calmodulin associated kinases.

**5'-Adenosine Monophosphate-Activated Protein Kinase (AMPK)**

Contraction mediated glucose uptake is strongly related to energy status of the cell.\textsuperscript{68} An increase in the adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio within the cell is known to be an activator of AMPK activity. AMPK is a
heterotrimer consisting of alpha (α), beta (β), and gamma (γ) subunits. The α subunit has an auto-inhibitory and catalytic domain, the β subunit binds glycogen which represses activity, and the γ subunit contains Batemans domains which binds AMP or ATP. The α, β, and γ subunits can be found in different isoforms: the α subunit can exist as the α₁ or α₂ isoforms; the β subunit can exist as the β₁ or β₂ isoform; and the γ subunit can exist as the γ₁, γ₂, or γ₃ isoforms. The dominant AMPK isoform expressed in human skeletal muscle tissue contains an α₂ catalytic subunit and the non-catalytic β₂ and γ₃ subunits.69-71 Activation of AMPK is achieved in many ways including, but not limited to, allosteric activation by AMP, allosteric activation of upstream kinases by AMP, AMP binding directly to AMPK, allosteric prevention of dephosphorylation by protein phosphatases, glycogen depletion, and nitric oxide (NO) activation of the α subunit. Activating factors include serine/threonine kinase 11 (LKB1), TGF-β activated kinase 1 (TAK1), calcium/calmodulin-dependent kinase kinase (CAMKK), NO, and higher AMP/ATP ratios while glycogen and protein phosphatase A act to depress activity.69 Activated AMPK, like Akt, phosphorylates AS160 and is a point of convergence between the insulin and contraction mediated pathways. Acutely polyphosphorylation of the RabGAP domain increases translocation of existing GLUT-4 protein stores.

AMPK also exerts a chronic effect on glucose uptake on skeletal muscle. The chronic effect (late phase: 24-48 hours) of AMPK on glucose uptake is realized through an increase in the number of GLUT-4 proteins available for translocation.19 That is, increased transcription of GLUT-4 proteins via direct phosphorylation of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) by activated AMPK.19
Chronologically, there is an increase in GLUT-4 messenger ribonucleic acid (mRNA), GLUT-4 protein, and insulin sensitivity. These effects are usually short lived, as within a week GLUT-4 protein number may be back to baseline. However, the effects are additive in that exercise multiple times throughout the week continues to increase the signal. Therefore, there is a cumulative effect of multiple stimuli.

**Calcium-Calmodulin Dependent Signaling**

Increases in intracellular calcium concentration have also been implicated in glucose uptake via downstream activation of targets calcium-calmodulin dependent protein kinase II (CAMKII), CAMKK, and protein kinase C (PKC). In skeletal muscle, increases in intracellular concentrations of calcium are fundamental to normal contraction physiology. The increase in intracellular calcium concentration facilitates greater binding of calcium to calmodulin, a protein when bound with calcium is capable of acting as second messenger and activating downstream targets such as CAMKK and CAMKII. CAMKII inhibition and CAMKK activation have both been shown to impact glucose uptake during muscle contraction. However, the role of CAMKK remains controversial as it can also activate AMPK which facilitates GLUT-4 mobilization and glucose uptake. In addition, AS160 contains a calmodulin binding domain that, when mutated, blunts contraction mediated glucose uptake.

**Nitric Oxide Mediated Glucose Uptake**

It has been proposed that there is also a third pathway for glucose uptake that is independent of both insulin and contraction mediated mechanisms. Sodium nitroprusside (SNP), a NO donor, significantly increases glucose uptake in the presence of wortmannin, a PI3K inhibitor, indicating that it is not a PI3K dependent mechanism. In addition, L-NG nitroarginine methyl ester (L-NAME), a nitric oxide synthase (NOS)
inhibitor, administration does not abolish contraction mediated glucose uptake in skeletal muscle. This suggests that the effects of NO are independent of both insulin and contraction and appear to augment glucose uptake systemically. Indeed, SNP treatment in rat skeletal muscle increases glucose uptake in a dose dependent manner and the mechanism responsible for the increase in glucose uptake has been attributed to translocation of GLUT-4. NO donors significantly increase the activity of AMPK by acting on the α1 catalytic subunit and AMPK can act in positive feedback loop with NOS. The increase in AMPK activity could explain the increase in NO mediated glucose uptake due to downstream signaling increasing GLUT-4 translocation and PGC-1α transcription. In addition, aminoimidazole carboxamide ribonucleotide (AICAR)-induced (AMPK agonist) GLUT-4 protein expression is NO dependent indicating a role for downstream NO signaling in AMPK mediated GLUT-4 protein expression as well. In resting muscle, NO stimulates glucose uptake through a cyclic guanosine monophosphate (cGMP)-dependent pathway that may involve cGMP-dependent kinase/protein kinase G (PKG) activation. Nitric oxide can also directly activate G protein α subunits, specifically the proto-oncogene p21ras for glucose transport, which increases glucose uptake through nuclear factor κB (NFκB) signaling. NFκB activity is increased transiently from treatment with NO donors and reacts rapidly to increase the activity of the extracellular signal-related kinases (ERK), c-Jun-NH₂ terminal kinase (JNK), and p38 subgroups of the mitogen-activated protein kinase (MAPK) family. However, the exact mechanism by which the MAPK subfamily elicits glucose uptake has not been elucidated. In addition to the acute effects of p38MAPK on glucose uptake, p38MAPK may also play a role in the chronic glycemic control as it
phosphorylates the transcription factor myocyte enhancer factor-2 (MEF2) which is implicated in the expression of GLUT-4 protein synthesis from the PGC-1α gene in skeletal muscle.\textsuperscript{82} p38MAPK may also play an acute role in glucose uptake, although the exact mechanism by which this increase is realized has not been elucidated. The role of nitric oxide in glucose uptake has been further supported by studies in which the administration of L-NAME results in marked hyperglycemia and insulin resistance. These findings support a role for NOS expression and activity in skeletal muscle as a major player in glucose handling.

An in vitro study by Lira et al. found that the NO donor S-nitroso-N-penicillamine (SNAP) increased GLUT-4 protein expression by 65% in L6 myotubes via a cGMP and AMPK-dependent mechanism.\textsuperscript{24} Moreover, L6 myotubes exposed to AICAR, an AMPK agonist, experienced a nine-fold increase in GLUT-4 mRNA.\textsuperscript{24} In vivo, GLUT-4 mRNA was increased 1.8 fold in the rat plantaris muscle 12 hrs after AICAR injection. Thus, evidence corroborates that in skeletal muscle, NO concentration likely influences GLUT-4 expression via control of cellular activity of AMPK kinases.

**Nitric Oxide**

The free radical NO is a ubiquitous signaling molecule that participates in virtually all cellular function in the human body. NO regulates cell metabolism, insulin signaling and secretion, vascular tone, and immune system function through interactions with thiol or transition metal centers in proteins or both.\textsuperscript{83} NO is a liable, cytotoxic molecule that acts primarily in a paracrine fashion with a half life of approximately 10 seconds in vivo.\textsuperscript{84} NO is rapidly oxidized forming nitrite, and subsequently nitrate by oxygenated hemoglobin, and ultimately excreted in the urine. NO is also susceptible to scavenging by other reactive oxygen species. NO is formed from the enzyme NOS. 3 different
isoforms of NOS exist: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). All three isoforms share a similar catalytic scheme in which the amino acid L-arginine is converted to NO. NOS agonists mediate NO release by activation of tyrosine kinase, PI3K activator. The activated PI3K then activates Akt which phosphorylates the eNOS Ser-1179 amino acid in a calcium/calmodulin dependent manner. The resulting reaction yields one NO molecule and the byproduct L-citrulline. NOS activity can be regulated at transcriptional, translational, and posttranslational levels. Therefore, bioavailability of NO is determined not only by reaction with other molecules (e.g. NO reaction with superoxide radical producing peroxynitrite) but also by NO synthesis from NOS. In humans, improved glucose tolerance and insulin sensitivity as seen with exercise intervention studies may be related to augmented expression of NOS in skeletal muscle. It has been well established that muscle tissue expresses NOS and there is significant NO production at rest from both constitutively activated forms found in skeletal muscle, nNOS and eNOS.

**Endothelial Nitric Oxide Synthase (eNOS)**

Within skeletal muscle eNOS is typically found in highest concentrations around the mitochondria and is thought to play the greater role in cell oxidative metabolism. Importantly, eNOS appears to play the greatest role in glucose handling as isolated skeletal muscle in eNOS knockout mice, in the absence of muscle perfusion, were found to have a 40% reduction in basal and insulin stimulated glucose uptake when compared to wild-type controls. This is supported by evidence that eNOS knockout animals are insulin resistant in addition to hypertensive and hyperlipidemic. The importance of NO production may be even more pronounced in T2DM as NOS inhibition decreases glucose uptake during exercise to a significantly greater degree in T2DM.
compared to healthy controls. This suggests a greater reliance on NO mediated glucose uptake in these individuals.

Another site of NO production is eNOS in the endothelial cells of the vasculature. NO produced in the vasculature is essential for relaxation of vascular smooth muscle cells, and in the regulation of angiogenesis, smooth muscle cell proliferation, leukocyte adhesion, platelet aggregation, and thrombosis. Endothelium derived NO is continuously synthesized from one of the guanidine-nitrogen atoms of the amino acid L-arginine in two successive reactions. The principle mechanism for the production of NO from eNOS is shear stress along the endothelial layer in the wall of the vasculature. eNOS agonists, such as acetylcholine and bradykinin, can also bind to receptors on endothelial cells leading to the release of NO. Both shear stress and agonist-mediated production of NO function by increasing endothelium intracellular calcium concentrations, which binds to the eNOS complex releasing an active eNOS protein. Controversy still exists as to whether or not NO of vascular origin affects skeletal muscle activity and function, but it is plausible.

Support for our enhanced external counterpulsation (EECP) hypothesis can be found in an animal model of external compression. Tan et al. applied intermittent pneumatic compression (IPC) (55 mmHg pressure achieved in <1 second) to the hind limbs of 16 male Sprague-Dawley rats for the durations of 0.5, 1.0, and 5 hours. Following IPC, eNOS in skeletal muscle homogenate was upregulated to 120%, 180%, and 270% from baseline, respectively. Similarly, nNOS expression was up-regulated, but to a lesser degree. Furthermore, EECP increased plasma levels of NO by 36% in coronary artery disease (CAD) patients following 35 1-hour sessions in a sham
controlled study. Indices of arterial and endothelial function were equally impressive after 35 1-hr sessions of EECP as endothelial-dependent flow mediated dilation (FMD), a non-invasive measure of NO bioavailability, in the brachial and femoral arteries was increased by 51% and 30%, respectively. These results, from this animal model resembling EECP, and from data in our own laboratory, serve to confirm our hypothesis that compression-induced improvement in vascular function is mediated by modulation of NO bioavailability and by regulating expression of NOS isoforms, in particular eNOS.

Neuronal Nitric Oxide Synthase (nNOS)

The mu neural isoform of NOS (nNOSµ) is the primary isoform found within skeletal muscle. In adult skeletal muscle, it is often found localized around sarcolemmal proteins that react to mechanical perturbations of the muscle. For example, nNOS is found in greatest quantities in the neuromuscular junction where the greatest strain is realized in skeletal muscle tissue. nNOSµ is constitutively active and is associated with the integrin and dystrophin complexes and is thought to be activated by movement of these cytoskeletal protein complexes. nNOSµ activity, and subsequently NO production, can be increased as much as 2-fold with contraction. nNOS appears to be the largest contributor to NO production in skeletal muscle (in non-inflammatory conditions). In humans, nNOS is expressed in both Type I and Type II muscle fibers.

L-Arginine is the substrate used for NO production and NOS activity is regulated by several mechanisms. L-arginine is competitively inhibited by asymmetric dimethylarginine (ADMA) and ADMA is degraded by dimethylarginine dimethylaminohydrolase (DDAH). DDAH is a redox sensitive protein that decreases its activity allowing for greater asymmetric dimethylarginine (ADMA) competition in more oxidized environments. BH4 is another redox sensitive cofactor for NOS. In addition,
calcium is a major player in NOS activity as increasing calcium concentrations contribute to greater NO production. nNOS protein expression is slightly elevated following IPC in underlying tissue following 1 hour of treatment.\textsuperscript{25}

**Calcium-Insensitive Nitric Oxide Synthase (iNOS)**

iNOS is a calcium insensitive isoform of NOS. iNOS is thought to only be activated when there is a great inflammatory response. In humans, iNOS mediated NO production plays the largest role in heart failure patients, auto-immune disorders, and acute inflammatory responses. iNOS appears to be upregulated in the presence of high concentrations of inflammatory cytokines. The activation of iNOS results in a high volume of NO production which is generally detrimental to the cell. However, in humans, iNOS appears to play a minimal role in cell signaling under normal physiological conditions. It has been suggested that iNOS downregulates GLUT-4 expression, but insulin has also been implicated in downregulation of iNOS at the post-transcriptional level.\textsuperscript{89, 90}

**Insulin and Nitric Oxide**

The vascular endothelium is the first organ that insulin encounters upon release from the beta cells of the pancreas. Not only is the endothelium involved in regulating the delivery of insulin and glucose to target tissues, it responds directly to insulin by producing NO. In vivo studies in human and animal models have shown that insulin stimulated production of NO from eNOS is a major player in insulin stimulated glucose uptake via increases in capillary recruitment and total limb blood flow in skeletal muscle.\textsuperscript{91} The capillary beds are more sensitive to insulin mediated NO production than the large conduit artery vessels greatly enhancing the delivery of insulin and glucose to the tissues that are responsible for whole body glucose uptake and disposal. Indeed,
the insulin effect to induce vasodilatation in the resistance arterioles in skeletal muscle is directly proportional to its ability to stimulate glucose uptake.\textsuperscript{92, 93} Braith et al. have shown that the reactivity of small the small resistance arterioles is improved in CAD patients following 35 1-hour sessions of EECP as peak forearm and calf blood flow increased 22\% and 19\% from pre-intervention values.\textsuperscript{94} Although speculative, increasing basal and insulin-mediated nitric oxide production in these resistance arterioles via EECP therapy may increase the potential for glucose uptake and disposal. Moreover, a rodent model of IPC acutely increases expression of vascular endothelial growth factor (VEGF), a protein that stimulates the growth of new blood vessels and potentiates an increase in capillary density and nutrient delivery.\textsuperscript{49}

**Reactive Oxygen Species**

Increased oxidative stress is an established contributor to the development and progression of diabetes.\textsuperscript{95} Diabetes, both type I and type II, is associated with increased production of free radicals and/or impaired antioxidant defenses, shifting redox balance toward greater oxidative stress.\textsuperscript{96, 97} Hyperglycemia has been shown to promote free radical generation through glucose autotoxidation\textsuperscript{98} and lipid peroxidation of low density lipoprotein (LDL) in a superoxide-dependent pathway.\textsuperscript{99} Free radical production due to hyperglycemia can also occur as glucose interacts with proteins ultimately resulting in the formation of advanced glycation endproducts (AGEs). These AGEs can promote free radical formation and quench and block anti-proliferative effects of NO through their binding with specific AGEs receptors. Davy et al. have shown that glycemic control correlated well with a marker of lipid peroxidation, 8-iso-prostaglandin 2α (8-iso-PGF-2α),\textsuperscript{100} but others have reported no correlation in Type I diabetics.\textsuperscript{101} Oxidative stress may play an important role in glycemic control as bioavailability of NO
may mediate NO mediated glucose uptake. Reduced availability of NO may occur due to an increase in breakdown by reactive oxygen species, especially superoxide which combines with NO to form peroxynitrite, a highly reactive molecule that markedly increases oxidative stress. EECP has been shown to decrease plasma levels of 8-iso-PGF-2α, viewed as the most valid plasma marker to assess systemic oxidative stress, by 21% in CAD patients. Although markers of oxidative stress following EECP therapy in patients with abnormal glucose tolerance have not been specifically investigated, improvements in angina and cardiac events in diabetics with CAD are similar to those with CAD alone. We hypothesized that systemic oxidative stress, as measured by 8-iso-PGF-2α, would be decreased in subjects with AGT following 35 1-hour sessions of EECP. Furthermore, the measure of 4-hydroxynoneal (4-HNE) protein conjugates in skeletal muscle biopsy samples was used as a marker biomarker of oxidative damage.

**Anti-Oxidants**

Anti-oxidants are substances, when present in a low concentration, capable of significantly delaying or preventing oxidation relative to an oxidizable substrate. Superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase are the major enzymatic antioxidants present in cells. Vitamins A, C and E as well as glutathione are some of the non-enzymatic endogenous enzymatic defenses. These anti-oxidants work in synergy with each other to defend against different types of free radicals. The most common antioxidant deficiencies reported in diabetics are lower levels of vitamin C, glutathione peroxidase and SOD. While the role of antioxidant defense in the treatment and pathology remains controversial, treatment of type T2DM patients with vitamin C or vitamin E decreased HbA1c levels, improved insulin action, decreased plasma insulin concentrations, and decreased indicators of oxidative
Measures of total anti-oxidant capacity in plasma (oxygen radical absorbance capacity assay), as well as quantification of SOD and GPX were performed to determine changes in anti-oxidant defense. We hypothesized that following 35 1-hour sessions of EECP there would be an increase in overall antioxidant capacity and/or a decrease in markers of oxidation. We hypothesized that these changes would correlate with indices of insulin sensitivity derived from the OGTT.

**Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC-1α)**

PGC-1α is a nuclear encoded transcriptional co-activator that increases the expression of several genes in skeletal muscle, including those involved in skeletal muscle metabolism and glycemic control. PGC-1α expression plays a large role in the metabolic phenotype as its expression is associated with production of proteins involved in oxidative metabolism, mitochondrial density, and fiber type. It also appears to be a site of integration of metabolic demand and adaptation as its expression is increased with cold exposure, fasting, and exercise. Additionally, PGC-1α expression has been shown to increase GLUT-4 and antioxidant enzyme expression. In diabetics, genes of PGC-1α were reduced and reductions in PGC-1α have been associated with elevated fasting insulin concentrations (indicative of decreased insulin sensitivity). In humans, PGC-1α is highly expressed in skeletal muscle tissue, although expression varies considerably between individuals and between fiber types, but is most highly expressed in slow oxidative fibers. PGC-1α is regulated by the transcription factors cAMP response element-binding protein (CREB), MEF-2, and activating transcription factor-2 (ATF-2). PGC-1α upregulation in response to exercise is thought to be mediated, in part, by augmented CAMKII and AMPK activity due to increased in calcium and glycolytic flux respectively. CAMKII and AMPK are capable of repressing
histone deacetylase 5 (HDAC5) association with the promoter MEF-2 and attenuating HDAC5’s repression of PGC-1alpha transcription. p38MAPK activity, which can be increased through calcium, AMPK, and NO-dependent signaling can also increase PGC-1α transcription through interaction with the MEF-2, CREB, and ATF-2 transcription factors. Not only does p38MAPK increases PGC-1α transcription, but it also enhances stability and increases the half life of the protein via dislodging the repressor myb-binding protein 1A (p160myb). Although numerous studies have shown an increase in PGC-1α expression due to exercise or stimulated muscle contraction, the effects of EECP have not been elucidated. We hypothesized that through alterations in nitric oxide mediated signaling and/or mechanical perturbations of skeletal muscle tissue in response to intermittent cuff compression PGC-1α expression would be upregulated. Ultimately, an increase in PGC-1α expression will increase GLUT-4 protein expression and potentiate a greater GLUT-4 protein ‘pool’ for translocation to the plasma membrane to facilitate glucose uptake. Indeed, modest over-expression of PGC-1α (~25%) increased GLUT-4 protein expression and insulin stimulated glucose transport in fast and slow rat skeletal muscle tissue.\textsuperscript{109} A single bout of exercise has been shown to increase PGC-1α expression as much as 2-fold in rodent skeletal muscle tissue.\textsuperscript{110, 111} Therefore, it is not unreasonable to conclude that EECP may increase PGC-1α expression through perturbations in metabolic flux, mechanical signaling, and/or nitric oxide bioavailability.

**Asymmetric Dimethylarginine (ADMA)**

Evidence suggests that ADMA is associated with endothelial dysfunction in a number of disorders, including, but not limited to, dyslipidemia, hyperhomocysteinemia, hypertension, CAD, heart failure, renal dysfunction, and T2DM.\textsuperscript{112, 113} ADMA, like NO, is
derived from the amino acid L-arginine. The production of ADMA from L-arginine can
decrease NO production due to substrate utilization. L-arginine concentration may be
even more important for glucose clearance as McConnel et al. have shown that L-
arginine infusion during cycling exercise increases glucose uptake in a NO dependent
manner.\textsuperscript{114} Since type II diabetics are even more reliant on nitric oxide mediated
glucose uptake, this may be of significant physiological relevance. ADMA biosynthesis
is catalyzed by the protein-arginine-N-methyltransferase (PRMTs) family of enzymes.
These enzymes utilize S-adenosylmethionine as a methyl group donor for methylation
of L-arginine residues on nuclear proteins. This process and the subsequent
proteolysis from nuclear proteins can result in three different free derivatives: N\textsuperscript{G} monomethyl-L-arginine (L-NMMA), symmetric dimethylarginine, and ADMA. While all of
these arginine derivatives are present in the human body, only ADMA appears to
biologically relevant.\textsuperscript{115, 116}

Following formation, free ADMA is released into the plasma where it can inhibit
NOS and decrease NO bioavailability leading to the development of endothelial
dysfunction.\textsuperscript{117} A study by Boger et al. demonstrated the negative linear relationship
between flow-mediated dilation (a measure of NO bioavailability) and ADMA plasma
concentrations in human subjects.\textsuperscript{116} In addition, inhibition of NOS impairs
microvascular recruitment and blunts insulin stimulated glucose uptake.\textsuperscript{118} In a study by
Steinberg et al., insulin resistance was associated with blunted endothelium-dependent
vasodilatation, but not endothelium-independent vasodilatation, during intrafemoral
artery infusions of sodium nitroprusside and metacholine chloride under euglycemic
hyperinsulinemic conditions.\textsuperscript{119} In addition, acute glucose challenge in mice and
exposure of cultured endothelial cells to high glucose increases the accumulation of ADMA.  \(^{120, 121}\)

**ADMA Metabolism**

Changes in plasma levels of ADMA in humans are mediated by an increase in production and/or a decrease in degradation. Increased ADMA concentrations in cultured endothelial cells, and in patients with endothelial dysfunction, are associated with increased reactive oxygen species production in supernatants and human plasma.  \(^{122, 123}\) One of the mechanisms by which ADMA concentration is increased is by oxidative stress. Oxidative stress is associated with an increase in PRMT activity and gene expression in the cell leading to an increase in ADMA formation.  \(^{116, 122-124}\) Boger et al., has shown that in human endothelial cells PRMT gene expression is upregulated in the presence of native LDL and oxidized LDL resulting in elevated levels of ADMA.  \(^{122}\) Furthermore, treatment with anti-oxidants abolished the rise in ADMA levels providing further evidence for the role of redox balance in the cell.  \(^{122}\) Tetrahydrabiopterin (BH\(_4\)), a cofactor for NOS, is also redox sensitive. Oxidation of BH\(_4\) to its inactive form BH\(_2\) renders it inactive and can lead to NOS ‘uncoupling’.  \(^{113, 115, 125}\) When ‘uncoupled’, electrons flowing from the reductase domain to the oxygenase domain in NOS are diverted to oxygen leading to the formation of superoxide. High levels of superoxide in the cell can react with NO leading to formation of peroxynitrite and further contributing to an increase in oxidants and a disturbance in redox balance. Evidence suggests that ADMA itself may also act to ‘uncouple’ NOS  \(^2, 4, 20\) or exhibit its effect purely through competition with L-Arginine.  \(^{113, 115, 125}\) This ‘uncoupling’ of NOS would magnify oxidative stress in a positive feedback fashion in the cell contributing further to endothelial and cell dysfunction in a ‘viscous cycle’.
While an increase in ADMA production due to oxidation of PRMTs is likely at times of high oxidative stress, elimination also plays a large role in explaining the elevation seen in those at cardiovascular risk. Humans produce about 300 μmol/day of ADMA during normal protein turnover with about 50 μmol/day excreted in the urine and 250 μmol/day metabolized by DDAH. ADMA is metabolized to form L-citrulline and dimethyamine by DDAH and impairment of DDAH activity may explain the potential for elevated levels of ADMA. Two isoforms of DDAH exist, DDAH I and DDAH II, with DDAH II being the most prevalent in tissues expressing endothelial NOS. DDAH is susceptible to inhibition/attenuation due to oxidative stress from oxidized LDL cholesterol, inflammatory cytokines, hyperhomocysteinemia, hyperglycemia and infectious agents. The sensitivity of DDAH to oxidative stress is conferred by reduction of a sulfahydryl group at the CYS-249 residue in the active site of the enzyme which inhibits ADMA metabolism. There is little doubt amongst researchers that the most common mechanism leading to accumulation of ADMA involves impaired metabolism by DDAH. In transgenic mice overexpressing DDAH, insulin sensitivity was enhanced during glucose challenge attributable to decreased plasma or tissue levels of ADMA.

Osanai et al. showed that ADMA levels in human endothelial cells in vitro decreased with 15 dynes/cm² of shear stress and DDAH activity increased at shear stress levels of > 25 dynes/cm². Braunth et al. showed that following 35 1-hour sessions of EECP in CAD patients that plasma concentrations of ADMA decreased by 28%. It is not unreasonable to conclude that DDAH activity may be increased with the magnitude of shear stress invoked with EECP (>200% of baseline, 49 vs. 23 dynes/cm²)
in a porcine model).\textsuperscript{26} Unfortunately, to date, there is not a reliable assay for humoral concentration of DDAH in humans. The mechanism by which EECP alters ADMA levels may be related to not only a decrease in production of ADMA, but an increase in degradation via preventing the oxidation of DDAH, eNOS and BH\textsubscript{4}.

**Inflammation**

Epidemiological evidence for an association of Type II diabetes and inflammation goes back as far as the 1950s. Increased levels of markers and mediators of inflammation such as high sensitivity C-reactive protein (hsCRP), interleukin 6 (IL-6), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) correlate well with incident T2DM.\textsuperscript{130} In addition, TNF-\(\alpha\) has been shown to cause insulin resistance in experimental models.\textsuperscript{131} Following EECP therapy, Braith et al. demonstrated plasma reductions in levels of TNF-\(\alpha\) (16\%), hsCRP (32\%), monocyte chemotactic protein 1 (MCP-1) (13\%) and soluble cell adhesion molecules (sVCAM) (6\%).\textsuperscript{27} Increased bioavailability of NO after EECP therapy is the likely mechanism responsible for the reduction in plasma inflammatory markers. NO serves an anti-inflammatory role by inhibiting the expression of MCP-1 and reducing VCAM-1 expression.\textsuperscript{132}

**Prostacyclin (PGI\textsubscript{2})**

In addition to NO, endothelial cells produce other vasodilators to regulate vascular tone and blood flow. Prostaglandins are a family of eicosanoids derived from endothelial cells. Shear stress has been shown to be a major stimulus for prostacyclin (PGI\textsubscript{2}) production.\textsuperscript{133} Once produced, PGI\textsubscript{2} diffuses into vascular smooth muscles and induces relaxation and ultimately vasodilatation via elevation of cyclic AMP levels. Decreased NO and PGI\textsubscript{2} release are well established in diabetics. This ultimately results in increased endothelial dysfunction. The potent endothelial-derived vasodilator
6-keto-prostaglandin 1α (6-keto-PGF1α), the stable metabolite of PGI₂, was increased by 71% following 35 1-hour sessions of EECP therapy in CAD patients.²⁷

**Vasoconstrictors**

Among the vasoconstrictors that are produced by endothelial cells is endothelin-1 (ET-1). Human ET-1 is derived from a reaction catalyzed by endothelin converting enzyme from the substrate big ET-1.¹³⁴ ET-1 exerts its major vascular effects, vasoconstriction and cell proliferation, through activation of ETₐ and ETₐ receptors on vascular smooth muscle cells. ET-1 is chronically upregulated in type II diabetes and hyperinsuleniemia may be a stimulus for ET-1 production from the endothelium.¹³⁵,¹³⁶ In a lean model of T2DM, Elgebaly et al. demonstrated that ETₐ and ETₐ blockade increased insulin sensitivity during hyperinsulinemic euglycemic clamps.¹³⁷ They also provided evidence that both ET receptors contribute to a decreased insulin-mediated vasodilatation in T2DM.¹³⁷ The opposing effect of ET-1 to NO is likely the culprit. EECP has been shown to decrease this potent vasoconstrictor by 25% in CAD patients.²⁷

Under normal physiological conditions, eicosanoids produced via the prostaglandin H synthase(PGHS)/Cyclooxygenase(COX) pathway generally induce vasodilatation. However, with endothelial dysfunction, PGHS-dependent vasoconstrictor production, specifically thromboxane A₂, may become more abundant. Thromboxane A₂ can act on their specific receptors in the vascular smooth muscle to produce vasoconstriction. Thromboxane A₂ has been indicated as a contributor to insulin resistance. Wang et al. demonstrated that exposure of cultured endothelial cells exposed to Thromboxane A₂ inhibited both basal- and insulin-stimulated Akt-Ser473
phosphorylation, Akt activity, the serine 1177 phosphorylation of endothelial NOS (eNOS-Ser1177), and angiogenesis.\textsuperscript{138}

Angiotensin II (Ang-II) is another potent vasoconstrictor found in the blood. In addition to its role in salt and water retention in the kidneys, Ang-II acts to directly influence endothelial function. The endothelium is capable of producing Ang-II via renin and/or angiotensin I uptake from the blood and conversion by locally active angiotensin converting enzyme (ACE). Once synthesized, Ang-II acts as a potent vasoconstrictor opposing the action of NO and PG\textsubscript{I2}. Furthermore, Ang-II is a major stimulator of NAD(P)H oxidase, a primary source of superoxide production that can contribute to oxidative stress. Although the mechanism of action has not been elucidated, angiotensin receptor blockers have been associated with a decrease in new onset diabetes.\textsuperscript{139}

**EECP**

EECP is a noninvasive, atraumatic, outpatient therapy that consists of three pneumatic compression cuffs applied to the calf, lower thigh, and upper thigh of each leg. These cuffs are sequentially inflated, from distal to proximal, with compressed air during the diastolic phase of the cardiac cycle and rapidly deflated in early systole. Inflation and deflation of the cuffs is triggered by events in the cardiac cycle via microprocessor interpreted electrocardiogram (ECG) signals. Acutely, EECP increases diastolic augmentation, reduces systolic afterload, and promotes venous return with a subsequent increase in cardiac output.\textsuperscript{140} EECP is traditionally used to treat symptomatic CAD patients who are not readily amendable for interventional procedures. Although initial theory as to the mechanism of action for angina reduction in CAD patients focused on angiogenesis, the literature points overwhelmingly to augmentation
of peripheral vascular function decreasing cardiac demand. The peripheral vascular nature of adaption to EECP therapy has lead to further indications for EECP treatment including, but not limited to, peripheral vascular disease and venous insufficiency. A standard course of EECP treatment is comprised of 35 1-hour sessions over a 7-week period.

Shear Stress

Among the primary mechanisms proposed for the salutary benefits of EECP therapy is shear stress. Shear stress is an eNOS agonist resulting in the synthesis and release of NO. The shear stress stimulus is transduced into the endothelial cell via the integrin/cytoskeleton mechanotransduction pathway. The sequential inflation (~300 mm Hg) of the 3 pneumatic EECP cuffs from calves to buttocks during a bout of EECP therapy produces a robust retrograde pressure wave in the femoral arteries and simultaneous moderate-pressure antegrade flow in the brachial arteries. In a porcine EECP model brachial artery blood flow was velocity was shown to increase by 132% and brachial artery wall shear stress increased by >200% during lower body compression of pneumatic EECP cuffs. It is not unreasonable to conclude that a significant alteration of blood flow velocity and shear stress is realized throughout the entire arterial tree, although it likely varies with location. Exercise is known to create pulsatile, hemodynamic shear forces that improve endothelial function, but exercise prescription adherence is low amongst Type II diabetics. In a sham controlled study of symptomatic CAD patients, Braith et al. have demonstrated that 35 1-hour sessions of EECP improve brachial and femoral artery flow mediated dilation (FMD), increases peak forearm and calf blood flow during venous occlusion plethysmography, increases plasma nitrate/nitrite (NOx) and 6-keto-PGF1a, and decreases ET-1, 8-iso-PGF2a,
TNF-a, hsCRP, MCP-1, and sVCAM. These results suggest that EECP improves peripheral arterial function by a shear stress mediated mechanism.

**Mechanical Effects of EECP**

While it has been well established that EECP causes an acute increase in blood flow to the muscle tissue, the effects of EECP on skeletal muscle have not been well characterized. A previous study by Crenshaw et al. has shown that application of external pressure to human cadaver limbs there is a linear relationship between external and intramuscular pressures (1988). The intramuscular pressure was not significantly different at different depths of the tissue, and limb circumference did not affect change in intramuscular pressure. Furthermore, in a rodent model of IPC, pressure underneath the cuffs closely resembles the pressure set on the compression unit. L6 myotubes that have been pressurized to mimic intramuscular pressures during walking or running showed a significant increase in succinate dehydrogenase (SDH) activity and glucose uptake with a decrease in lactate release. SDH enhancement, an indicator of tricarboxylic acid cycle (TCA) activity, increases the capacity to degrade lactate and glucose. Although the mechanism by which external pressure induces aerobic metabolism has yet to be elucidated, this suggests that mechanical pressure devoid of active contraction can increase metabolism and potentially alter AMP/ATP ratios. Indeed, in an acute rodent model of IPC, the arterial venous difference was shown to increase during compressions. This is indicative of oxygen consumption and supports an increase in tissue metabolism during external compression. Furthermore, a study by Grayson et al., demonstrated a significant increase in somatic oxygen consumption during 30 minutes of EECP therapy. The observed increase in oxygen consumption was not significantly different between CAD
and healthy subjects. Interestingly, all NOS isoforms may be regulated by hypoxia.\textsuperscript{83} The intermittent hypoxia experienced during cuff compression may upregulate NOS and contribute to greater metabolism through nitric oxide mediated cell signaling. Calcium transients may also be induced during EECP therapy as C2C12 myotubes subjected to compression demonstrate an increase in intracellular calcium concentrations.\textsuperscript{143} The exact mechanism by which calcium is released is unclear, but pressure sensitive calcium channels or molecular signaling could play a role. Another potential mechanism for the perturbation in skeletal muscle metabolism during IPC is stretch of the muscle tissue directly under and in the immediate vicinity of cuff edges. Indeed, NO production increases approximately 20\% in isolated soleus muscle from a single stretch.\textsuperscript{53} Furthermore, myotubes subjected to cyclic stretching for 2 hours showed a 42\% increase in NOS activity from basal rates. Perhaps of long-term consequence of EECP compression, mechanical loading may upregulate nNOS expression as a result of membrane associated proteins responsive to mechanical disruption. Mechanical loading has been shown to regulate expression of talin in C2C12 myotubes\textsuperscript{53} and nNOS protein expression is slightly elevated following IPC in underlying tissue following 1 hour of treatment.\textsuperscript{25}

The skeletal muscle response to 35 1-hour sessions of EECP is uncharacterized. However, potential mechanisms for alteration of skeletal muscle metabolism in the short and long term exist. EECP should elicit an increase in intramuscular pressure and stretch the compressed limbs. In addition, acute aerobic metabolism appears to be augmented as demonstrated by an increase in SDH activity, glucose uptake, and oxygen consumption following a single session of IPC. Indeed, Lira et al. examined the
regulation of PGC-1α in muscle cells and found that NO donors induce PGC-1α expression via activation of the α1 isoform of AMPK. Further, chronic NO treatment causes increased mitochondrial volume and increased rates of basal and uncoupled aerobic respiration in muscle cells; an effect that is prevented by inhibition of AMPK activity. These data support the conclusion that increased NO availability in skeletal muscle increases AMPK-PGC-1α signaling, resulting in improved metabolic function.

**Preliminary Data**

Given the strong evidence that NO bioavailability and limb perfusion is dramatically improved by EECP, we designed a pilot study to determine if 35 1-hour sessions of EECP would have a beneficial chronic effect on fasting glycemic control in patients with T2DM. To the best of our knowledge, the only previous study to measure the salutary effects of EECP on blood glucose was an acute investigation performed immediately following a single EECP session. In that study, blood glucose was acutely reduced following one session of EECP in 18 men and 4 women with diabetes (148 to 129 mg/dL; p<0.0001). We recruited 10 subjects with T2DM (n=10 males; age = 65 ± 9 years; weight = 215 ± 40 lbs) and measured fasting blood glucose levels and performed oral glucose tolerance tests (OGTT; 75 grams of glucose) before and after 35 sessions of EECP. Subjects fasted and withheld antiglycemic medications for 12 hours prior to measurement of blood glucose. Post-intervention laboratory studies were performed between 48 to 72 hours after the last EECP session in an attempt to “capture” the chronic effect. We found that EECP significantly reduced fasting glucose by 20% (198 ± 20 to 159 ± 16 mg/dL; p = 0.02). EECP also significantly improved the 2- hour changes in plasma glucose (blood samples at 30, 60, 90, and 120 minutes) during the OGTT (185 ± 35 to 146 ± 20 mg/dL; p = 0.04). We interpret our pilot data as
strong support for the hypothesis that EECP may improve chronic glycemic control in patients with T2DM. We speculated that improved NO bioavailability and skeletal muscle perfusion were the mechanisms underlying sustained improvements in glycemic control.
CHAPTER 3
MATERIALS AND METHODS

This proof-of-concept study was designed to be a single-center, prospective, randomized, and controlled investigation to determine the efficacy of enhanced external counterpulsation (EECP) as therapy to improve glycemic control in patients with abnormal glucose tolerance (AGT). Eighteen (n=18) patients with AGT were recruited by advertisement. Subjects were randomized to receive either 35 1-hour sessions of EECP with target inflation pressure of 300 mm Hg per cuff (EECP group; n=12) or continued medical care with no EECP intervention (Time-Control group; n=6). Laboratory testing was performed at study entry, and after 35 1-hour EECP sessions (7 weeks) or matched control period. All testing and EECP intervention sessions occurred in the Center for Exercise Science at the University of Florida. A time and events table is outlined in Table 2-1.

Group Assignment

After signing an informed consent document, patients who were eligible, based on inclusion and exclusion criteria and screening, were randomized in a 2:1 ratio between a group that received 35 1-hour sessions of EECP (n=12) or to a ‘Standard Care’ control group (n=6). Subjects were instructed to refrain from initiating structured exercise training programs during the study.

Eligibility Criteria

Inclusion Criteria

All subjects enrolled in the study were required to meet the following inclusion criteria: 1) written informed consent for participation in the study was given; 2) presence of abnormal glucose tolerance (fasting plasma glucose >110 mg/dL and a plasma
glucose concentration of 140 to 199 mg/dL after a 2-hour OGTT or T2DM as determined through clinical diagnoses and screening).

**Exclusion Criteria**

Potential subjects were excluded from the study for any of the following reasons: 1) insulin dependence for glycemic control; 2) any major illness in the prior 3 months; 3) previous treatment with EECP; 4) participation in moderate intensity exercise for 20 minutes, 2 or more times per week; 5) history of deep vein thrombosis, phlebitis, stasis ulcer and/or pulmonary embolism; 6) aged less than 21 years; 7) aged greater than 75 years; 8) pregnancy; 9) uncontrolled hypertension (defined as a systolic blood pressure of 180 mmHg or more and/or a diastolic blood pressure of 110 mmHg or more, measured as the average of at least two readings, obtained at different occasions); 10) systemic hypotension; 11) any medical, psychological, cognitive, social or legal condition that would interfere with the ability of the subject to give informed consent and/or his or her capacity to comply with all study requirements, including the necessary time commitment; 12) cardiac arrhythmia that would significantly interfere with the triggering of the EECP device; 13) acute coronary syndrome such as unstable angina or acute myocardial infarction.

**EECP Methods**

Patients in the EECP group (n=12) were treated with EECP for 1 hour daily on Monday through Friday for 7 consecutive weeks, resulting in a total of 35 hours of EECP. All patients were monitored clinically and hemodynamically, by plethysmography, oximetry and electrocardiographic monitoring, during EECP treatment. EECP involves sequential inflation and deflation of compressible cuffs wrapped around the patient’s calves, lower thighs, and upper thighs. Compressed air
pressure is applied via the cuffs to the lower extremities in a sequence synchronized with the cardiac cycle via microprocessor-interpreted electrocardiogram (ECG) signals. The diastolic augmentation pressure is progressively increased by increasing external compression. In this study, the pressure applied to the cuffs during EECP was set at 300 mmHg. Blood pressure changes and diastolic augmentation were continuously monitored by finger plethysmography. To assess the hemodynamic effect of EECP, two ratios were computed electronically, using the systolic and diastolic peak pressures or the area under the systolic and diastolic curves. Ratios greater than 1.0 correspond to diastolic values greater than systolic values. An effectiveness ratio of 1.5 to 2 is associated with an optimal increase in diastolic femoral artery and aortic retrograde flow, and brachial artery antegrade flow.\textsuperscript{145} Optimal pressure for modulation of glucose metabolism is unknown.

**Screening (Fasting Plasma Glucose and Blood Pressure)**

After informed consent was provided, screening consisted of a simple finger-stick measurement of plasma glucose concentration using an at-home blood glucose meter (Accu-Check\textsuperscript{\textregistered} Advantage, Roche Diagnostics) following an overnight fast. Participants who had a fasting blood glucose of >110 mg/dL were given the opportunity to continue in the study. Additionally, resting blood pressure measurements were performed by an experienced technician using a standard manual mercury sphygmomanometer. Subjects with uncontrolled hypertension defined as a systolic blood pressure of 180 mmHg or more and/or a diastolic blood pressure of 110 mmHg or more were excluded from the study.
Dual Energy X-Ray Absorptiometry (DEXA)

Subjects underwent a DEXA scan at study entry and after 35 1-hour sessions of EECP or the control period. The DEXA is a low radiation device which measures body composition and bone mineral density. There is a small x-ray dosage during the body composition measurement. The effective radiation dose equivalent (HE) is about 2.5 uSv during a total body scan. This exposure is approximately 0.3% of the average annual per capita background radiation exposure in the U.S (equivalent to 1.1 days of natural background exposure). DEXA scans were performed by Jeffrey S. Martin (Florida Department of Health License #BMO 68895) in the Center for Exercise Science at the University of Florida.

Skeletal Muscle Biopsies

Subjects were asked to report to Dr. Braith’s Cardiovascular Laboratory in the Center for Exercise Science at the University of Florida where skeletal muscle biopsies were performed. Biopsies were performed at study entry and after 35 1-hour sessions of EECP or the control period. Muscle biopsies commenced within 24-48 hours after the last EECP therapy session, if subjects were in the treatment group, in an effort to capture the chronic adaptation to EECP therapy as opposed to the acute effect of the last session. The muscle biopsies were performed by Juan Aranda Jr., M.D. Skeletal muscle tissue (approximately 150 mg) was extracted from the right vastus lateralis of each subject using a percutaneous needle under local subcutaneous anesthetic (1% lidocaine) using a modification of the Bergstrom technique.\textsuperscript{146, 147} Two-thirds (approximately 100 mg) of the muscle biopsy sample was immediately snap frozen in liquid nitrogen and stored at -80°C for quantitative protein analysis via western blotting. The other third of the muscle biopsy sample (approximately 50-60 mg) was separated
for cryo-sectioning. After assuring alignment of muscle fibers at resting length, this portion was frozen in optimal cutting temperature (OCT) medium by liquid nitrogen cooled isopentane and stored at -80°C until cryo-sectioning.

**Oral Glucose Tolerance Tests**

Oral glucose tolerance tests (OGTT) were performed in all subjects at study entry and after 35 1-hour sessions of EECP or Time-Control. During the 3 days prior to each clamp, subjects were instructed to consume a standardized diet, consisting of at least 200 grams of carbohydrate per day, while abstaining from caffeine and alcohol. Subjects reported to the laboratory in the morning following an overnight fast and withheld vasoactive medications for 10-12 hours and glycemic control medications for at least 24 hours. A catheter was placed in a vein in the anticubital space. 6 mL blood samples were drawn at -10, -5, and 0 minutes to account for the pulsatile nature of insulin secretion. An average of the 3 baseline time-points was used to determine fasting insulin and glucose concentrations. Following ingestion of a glucose beverage (Fisherbrand® Glucose Tolerance Test Beverage, 7.5g glucose/fl oz., 10 oz. beverage, 75 grams of glucose ingested), 6 mL blood samples were taken at 30, 60, 90, and 120 minutes from the time the drink was finished. Subjects were instructed to drink the entire beverage as quickly as possible and in a time not to exceed 3 minutes. Blood samples were evaluated by commercially available assays for plasma glucose and insulin concentrations.

**Blood Sampling**

In addition to the blood samples collected for plasma insulin and glucose concentrations; approximately 20 mL of blood samples were collected from all subjects at study entry, and after 35 1-hour sessions of EECP (7 weeks) or matched control
period. Venous blood was collected from a vein of the anticubital space, using the same Teflon catheter as described in the oral glucose tolerance testing. These blood samples were drawn at the -10 minute time-point prior to the start of OGTT. Blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately underwent centrifugation at 3,000 rpm for approximately 15 minutes. Plasma was immediately aliquoted into sterile tubes and stored at -80° C for analysis at completion of the study.

**Peripheral Flow Mediated Dilation (FMD)**

The FMD technique was used to determine endothelial-dependent reactivity in the brachial and popliteal arteries. At study entry and within 24-48 hours after the final EECP therapy treatment or time matched control, subjects fasted for at least 8 hours and withheld all vasoactive medications for 10-12 hours and reported to Dr. Braith’s Vascular Laboratory. After lying quietly for 15 minutes, a 10.5MHz linear phase array ultrasound transducer (ATL HDI® 3000; Advanced Technologies) was used to image the right brachial artery longitudinally. Resting baseline end diastolic brachial diameters and blood velocity were obtained with the transducer placed 3-5 cm above the anticubital fossa. After obtaining baseline diameter measures, reactive hyperemia was produced by inflating a blood pressure cuff placed on the upper forearm, 1-2 cm below the elbow, for 5 minutes at 200 mmHg. The transducer was manually held in the same position for the duration of cuff inflation. Immediately following cuff release, brachial artery blood flow velocity was measured for 20 seconds. Brachial artery diameter was then imaged and recorded for an additional 2 minutes. Ultrasound images were recorded directly to a digital storage device via video interface (Pinnacle, Avid®)
Technology) for off-line electronic image analysis using automated FMD software (Vascular Research Tools™; Medical Imaging Applications LLC, Iowa).

Brachial artery diameters were determined during end-diastole (gated with electrocardiogram R wave) by measuring the distance between the near and far wall of the intima. Brachial FMD was calculated in absolute (mm) and relative (FMD%) peak change in brachial artery diameter in response to the hyperemic stimulus. Brachial measurements are normalized to the mean shear rate calculated from the first 10 seconds following cuff dilation. With the use of artery diameter and mean velocity doppler measurements, blood flow in the brachial artery was calculated using the following equation: blood flow (mL/min) = mean velocity \cdot \pi \cdot (diameter/2)^2 \cdot 60. Additionally, in the absence of blood viscosity, shear rate is measured by the following equation: shear rate (s\(^{-1}\)) = 4 \cdot \text{mean blood velocity (cm/s)} \cdot \text{diameter (cm}\(^{-1}\)).

Popliteal artery FMD was performed at the popliteal fossa, 2 to 3 cm above the bifurcation, using the approximate FMD protocol described above for brachial FMD.

**Venous Occlusion Plethysmography (VOP)**

**Forearm and Calf Blood Flow**

To determine EECP-mediated adaptation in small resistance arteries, calf blood flow (CBF) and forearm (FBF) responses were determined independently by venous occlusion plethysmography (EC-6™, D.E. Hokanson, Inc.) using calibrated mercury strain-gauges. For CBF, strain gauges were applied to the widest part of the non-dominant calf. Patients rested supine for 20 minutes with legs elevated above the right atrium to achieve stable baseline measurements of CBF. To measure CBF, a thigh cuff was inflated to 50 mmHg for 7 seconds every 15 seconds for 3 minutes using a rapid cuff inflator to prevent venous outflow. One minute before measurements, an
ankle cuff was inflated to constant pressure 50 mmHg above systolic pressure to occlude ankle circulation during CBF measurements. The CBF output signal was transmitted to NIVP3 software program loaded on a desktop computer and expressed as milliliters (mL) per minute per 100 mL of calf tissue (mL·min$^{-1}$·100 mL$^{-1}$ tissue). CBF for one minute is the average of one plethysmographic measurement every 15 seconds for one minute.$^{151,152}$

To assess FBF the mercury strain gauge was placed 5mm below the anitcubital space and cuffs were placed on the upper arm and wrist. Patients rested supine for 20 minutes with the arm elevated to level of heart, parallel with the body. The upper arm cuff was inflated to 50 mmHg for 7 seconds every 15 seconds using the rapid cuff inflator to prevent venous outflow. One minute prior to measurements, the wrist cuff was inflated to a constant pressure of 50 mmHg above systolic pressure to occlude hand circulation during CBF measurements. The output signal is again transmitted to the NIVP3 software program and calculations are performed in the same manner as CBF.

**Forearm Flow During Reactive Hyperemia**

Endothelium-dependent FBF was measured following 5 minutes of upper arm arterial occlusion during reactive hyperemia of the forearm.$^{151,152}$ Endothelium-dependent vasodilation (EDV) during reactive hyperemia in the forearm has been shown to correlate highly with acetylcholine-induced EDV in patients with essential hypertension.$^{148,153}$ Therefore, it is a good non-invasive measurement of EDV of resistance vasculature. Blood pressure cuffs were placed on the upper arm, 5 cm above the anticubital fossa, but below the venous occlusion cuff. After baseline FBF was confirmed to be stable for 2 minutes and recorded, the upper arm cuff was rapidly
inflated to 200 mmHg for 5 minutes and then released. At 4 minutes of occlusion, the wrist cuff was rapidly inflated to 200 mmHg. Peak FBF was recorded as the highest FBF observed immediately following releases of the cuff, and total FBF was recorded as the area under the time-curve after baseline FBF is subtracted. Cuff inflation pressures and timing following occlusion were identical to those used for resting blood flow measurements.

**Calf Flow During Reactive Hyperemia**

Endothelium-dependent CBF were measured following 5 minutes of upper leg arterial occlusion during reactive hyperemia of the calf. A blood pressure cuff was inflated on the upper thigh above the knee, but below the venous occlusion cuff. After baseline CBF was confirmed to be stable for 2 minutes, the thigh cuff was rapidly inflated to 200 mmHg for 5 minutes and then released. Peak CBF was recorded as the highest CBF observed immediately following release of the cuff, and total CBF recorded as the area under the time-curve after baseline CBF is subtracted. Cuff inflation pressures and timing following occlusion were identical to those used for resting blood flow measurements.

**Western Blotting**

Frozen muscle samples were homogenized using procedures as reported by Sakamoto et al. Briefly, samples were weighed and placed in the appropriate volume of homogenate buffer containing protease inhibitor (Thermo Scientific, 78415) and phosphatase inhibitor (Thermo Scientific, 78420) cocktails and rotated end over end for 1 hour at 4°C. Samples were then sonicated three times for 10 seconds each. Following sonication, samples were centrifuged at 14,000 rcf for 10 minutes at 4°C and supernatant was transferred to polypropylene tubes and stored at -80°C. Protein
concentrations were measured using the DC™ Protein Assay Kit (Bio-Rad, 500-0116). Aliquots of muscle homogenate (50µg and 75µg) were separated in 4-20% SDS-PAGE gels, transferred to nitrocellulose membranes, and stained with Ponceau S to verify transfer. Separate protein blots were probed for proteins of interest along with β-actin as a loading control. The primary antibodies used were: goat anti-Akt1/2 (N-19) (Santa Cruz Biotechnology; sc-1619), rabbit anti-phospho-AKT1/2/3 (Ser473) (Santa Cruz Biotechnology; sc-7985-R), mouse anti-AMPKα (Cell Signaling Technology; 2793), rabbit anti-phospho(Thr172)-AMPKα (Millipore;07-681), rabbit anti-TBC1D4 (AS160) (Abcam; ab24469), rabbit anti-phospho (Thr642)-TBC1D4 (AS160) (Novus Biologicals; NBP1-44074), goat anti-GLUT-4 (Santa Cruz Biotechnology;sc-1608), mouse anti-eNOS(6H2) (Cell Signaling Technology; 5880), rabbit anti-nNOS (Cayman;160870), mouse anti-SOD-2 (B-1) (Santa Cruz Biotechnology; sc-133254), mouse anti-SOD-1 (G-11) (Santa Cruz Biotechnology; sc-17767); mouse anti-GPx-1/2 (E-7) (Santa Cruz Biotechnology; sc-74498), rabbit anti-4-HNE (Abcam;ab46545), and rabbit anti-β-actin (Abcam;ab8227). Blots were blocked with Odyssey® blocking buffer (LI-COR Biosciences; 927-40000) before incubation with primary antibodies. Incubations with secondary antibodies IRDye® 680CW donkey anit-goat (LI-COR Biosciences; 926-32224), IRDye® 680CW donkey anti-mouse (LI-COR Biosciences; 926-32222), IRDye® 800CW donkey anti-mouse (LI-COR Biosciences; 926-32212), IRDye® 800CW donkey anti-rabbit (LI-COR Biosciences; 926-32213 ) were performed. Protein blots were scanned and proteins of interest detected using the Odyssey® infrared imaging system (LI-COR Biotechnology, Lincoln, NE). Some membranes were stripped using 10 mL of Restore™ western blot stripping buffer (Thermo Scientific; 21059) at room temperature.
for 60 minutes. Following stripping, membranes were washed in TBS 4 times for 5 minutes each wash. After washing, membranes were read using the Odyssey infrared imaging system to assure complete stripping of previous signals/proteins before probing for additional proteins of interest.

**Immunostaining for Capillary Density**

Frozen sections of muscle biopsy tissue were cut (8 µm) in a cryostat on fixed on microscope slides. Slides were then allowed to come to room temperature, and fixed in Carnoy’s fixative (60% ethyl alcohol, 30% chloroform, and 10% glacial acetic acid) for 10 minutes. After rinsing with several exchanges of dH₂O, slides were incubated in 1% amylase at 37°C for 60 minutes. Following another rinse, slides were oxidized in 0.5% periodic acid (Sigma-Aldrich; P-7875) for 10 minutes. Again, slides were rinsed with several exchanges of dH₂O, followed by incubation in Schiff’s reagent (Sigma-Aldrich; 3952016) for 5 minutes. Slides were then rinsed and dehydrated in ascending concentrations of ethyl alcohol and mounted with aqueous mounting medium. Images of the muscle sections were captured with an inverted microscope (Olympus America; Center Valley, PA). Stained sections were analyzed by magnifying and projecting numerous artifact-free sections of approximately 0.20 mm² areas onto a screen. The number of fibers within the known area were counted and capillary density (capillaries/mm²), capillary/fiber ratio, and mean fiber area was calculated. Number of fibers and capillaries was determined on 166 ± 13 fibers per biopsy. Areas were assessed by manual drawing of the perimeter using the National Institutes of Health’s public software (Image J, NIH, USA).
Biochemical Assays

Vasodilator Measurements

Because NO is rapidly converted to nitrite and nitrate (NOx) in plasma, measurement of these metabolites can be used to estimate NO production. Since plasma NOx can be influenced by dietary nitrates, subjects were asked to follow the National Institutes of Health low nitrate diet guidelines for 36-48 hours prior to blood sampling. Plasma NOx was measured using a commercially available assay kit (Cayman Chemical, Inc.) that converts all nitrate to nitrite using NADH-dependent nitrate reductase. Spectrophotometric analysis of total nitrite was then performed using Greiss reagent and the absorbance measured at 540 nm.

The major metabolite of the vasodilator prostacyclin, 6-keto-PGF1α, was also measured by commercially available enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Inc.).

Vasoconstrictor Measurements

Commercially available competitive ELISA kits were used to determine plasma endothelin (ET-1) (R&D Systems, Inc.). The ET-1 ELISA kits utilize a microplate that has been pre-coated with an antibody specific for ET-1. ET-1 present in plasma samples is sandwiched by the immobilized antibody and the enzyme-linked antibody specific for ET-1. Tetramethylbenzadine (TMB) is added to the microplate wells and absorbance at 450 nm is proportional to the amount of ET-1 present in plasma samples.

Lipid Peroxidation

Isoprostanes are important products of lipid peroxidation, and their measurement has emerged as one of the most reliable approaches to assess oxidative stress in vivo. Limited amounts of isoprostanes can be absorbed and diet has a limited effect on
plasma levels of these compounds.\textsuperscript{156} Therefore, oxidative stress induced lipid peroxidation was assessed by measuring plasma levels of 8-iso-prostaglandin 2α (8-iso-PGF\textsubscript{2α}) using a commercially available ELISA kit (Enzo Life Sciences). This assay is based on the competition between 8-iso-PGF\textsubscript{2α} and an 8-iso-PGF\textsubscript{2α} acetylcholinesterase conjugate for a limited number of 8-iso-PGF\textsubscript{2α}-specific rabbit antiserum binding sites. The amount of 8-iso conjugate that is able to bind is inversely proportional to the amount of free 8-iso present in the sample. The plate is read at 405 nm on a spectrophotometer.

**Antioxidant Capacity**

Total antioxidant capacity was measured using a Trolox-equivalent antioxidant capacity (oxygen radical absorbance capacity, ORAC) assay kit provided by ZenBio, Inc. This assay relies on the ability of antioxidants in the plasma sample to inhibit the oxidation of 2,2'-Azobis-2-methyl-propanimidamide, dihydrochloride(AAPH) by peroxyl-radical formation. The ORAC assay is a kinetic assay measuring fluorescein decay and antioxidant protection over time. The antioxidant activity is normalized to Trolox units to quantify antioxidant capacity.

**ADMA**

Serum levels of the endogenous eNOS competitive inhibitor, asymmetric dimethylarginine (ADMA) were measured using an ELISA kit (Alpco™, Inc.). Samples were treated with a derivatization-reagent for ADMA coupling and incubated in microplates pre-coated with ADMA-derivative tracer. ADMA present in plasma samples competes with the tracer immobilized in the wells for binding of the polyclonal antibodies. Therefore, the concentration of tracer-bound antibody as measured by
spectrophotometry at 450 nm, is inversely proportional to ADMA concentration in the sample.

**Inflammatory Markers**

High sensitivity CRP (hsCRP) was determined by a commercially available ELISA (BioQuant). The hsCRP assay is a solid phase direct sandwich assay. The samples and anti-CRP antibodies conjugated with horseradish peroxidase are added to wells pre-coated with monoclonal antibodies for CRP. CRP in the serum binds to the anti-CRP monoclonal antibodies and the second antibody then binds to CRP. Ultimately, after addition of substrate, the plate is read at 450 nm on a spectrophotometer with the absorbance being proportional to the CRP in the serum samples.

Similarly, tumor necrosis factor-α (TNF-α) (Cayman Chemical, Inc.) was measured using a sandwich ELISA and its absorbance read at 405 nm using a spectrophotometer.

**Glycosylated Hemoglobin (HbA1c)**

Plasma samples were transported to Shands Hospital Clinic Laboratories at University of Florida CORE lab for HbA1c analysis by standard procedures. Samples were kept on ice and transported to the lab within 30 minutes of blood sampling. Analysis was performed using conventional clinical techniques.

**Vascular Endothelial Growth Factor (VEGF)**

VEGF was measured by commercially available ELISA kits (R&D Systems). Microplates pre-coated with a monoclonal antibody specific for VEGF will bind and immobilize VEGF present in plasma samples. Ultimately, the substrate is added to microplate wells after the addition of a secondary antibody which will increase color in
proportion to the amount of VEGF present in the plasma samples. Absorbance is read on a spectrophotometer at 540 nm.

**Insulin**

Plasma insulin was measured by commercially available ELISA kits (Alpcô™ Diagnostics). The insulin ELISA is a sandwich type assay in which a microplate is pre-coated with a monoclonal antibody specific for insulin. After the addition of samples with a horseradish peroxidase enzyme labeled monoclonal antibody to the microplate wells, the microplate is incubated and washed with wash buffer. TMB is added to the microplate, incubation commences, and finally stop solution is added to the wells. Absorbance is read on a spectrophotometer at 450 nm and is proportional to plasma insulin concentration present in the sample.

**Glucose**

Plasma glucose was quantified using a commercially available assay kit (Cayman Chemical, Inc.). The glucose assay utilizes the glucose oxidase-peroxide reaction. Glucose is oxidized to δ-gluconolactone with concomitant reduction of the flavin adenine dinucleotide (FAD)-dependent enzyme glucose oxidase. The reduced form of glucose oxidase is regenerated to its oxidized form by molecular oxygen to produce hydrogen peroxide (H₂O₂). Finally, with horseradish peroxidase as a catalyst, H₂O₂ reacts with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminoantipyrine to generate a pink dye with absorption read at 515 nm on a spectrophotometer. Absorbance is proportional to the amount of plasma glucose concentration present in samples.
Statistical Considerations

Statistical Analysis

This study was designed as an open label, unbalanced, randomized study of EECP vs. Standard of Care for patients with AGT. All data were tested for normal distribution using the Shapiro-Wilk test for normality. An alpha level of $P < 0.05$ was required for statistical significance. Satterthwaite corrected two sample t-tests of baseline subject characteristics were performed, and $P$-values reported for each variable. A repeated measures 2-way analysis of variance was used to evaluate the continuous primary dependent variables associated with, brachial artery FMD, popliteal artery FMD, forearm VOP, calf VOP, capillarity, plasma markers, fasting indices of glycemic control, and dynamic measures of glucose tolerance. When a significant group-by-time interaction was observed, within-group comparisons between time points and between-group comparisons at each time point were performed using Bonferroni’s post hoc test for pairwise comparison. To achieve an overall family error rate of 5% for between-group comparisons, $\alpha$ was adjusted for multiple comparisons. When comparing between groups at each time point, $\alpha$ was adjusted for 2 comparisons, $0.05/2 = 0.025$. Western blot proteins of interest were analyzed using the Satterthwaite corrected two sample t-test of percent change from baseline to week 7 as our dependent variable, and study group (EECP vs. usual care) as our independent variable. All statistical analyses were performed using IBM® SPSS® Statistics 19 for Windows (Chicago, IL). All data are reported as mean ± SEM.

Power Analysis

A power analysis based on a sample size of 15 (n=10 EECP Group and n=5 Control Group) was performed to estimate the statistical power related to testing the
following hypothesis (stated as null): 1) The distribution of change in average insulin sensitivity determined from oral glucose tolerance test using the Matsuda Index, when compared to standard care from Baseline to Week 7 is the same for EECP and Standard of care and 2) The distribution of change in GLUT-4 protein in muscle from Baseline to Week 7 is the same for EECP and standard care.

Based on the data of O'Gorman et al., we projected differences for Hypothesis 1 of 4.1 [SD=SE*√(n)=2.0] and 2.6 mg/kg/min (SD=1.0=about half of 2.0 as changes in controls will be more stable) for EECP and control subjects, respectively. A study of 10 evaluable EECP subjects and 5 evaluable controls will have 80% power, based on the Satterthwaite corrected t-test to have a $P$-value below 5% two-sided.

For Hypothesis 2, the means were anticipated to be 0.35 and 0.2 U for EECP and standard care subjects, respectively. Using calculations as above for Hypothesis 2, the anticipated standard deviation were about 0.17 for the EECP group and about 0.085 for the control group. The study of 10 evaluable EECP subjects and 5 evaluable controls will have 90% power, based on the Satterthwaite corrected t-test to have a $P$-value below 5% two-sided.
Table 3-1. EECP and glycemic control protocol; time and events

<table>
<thead>
<tr>
<th>Events</th>
<th>Consent and Screening (V1,V2)</th>
<th>V3</th>
<th>V4</th>
<th>V5- V39</th>
<th>V40</th>
<th>V41</th>
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<tr>
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<td>EECP</td>
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</tbody>
</table>

V = Visit; DEXA = Dual energy x-ray absorptiometry; OGTT = oral glucose tolerance test; VOP = venous occlusion plethysmography; FMD = flow mediated dilation; EECP = enhanced external counterpulsation
A total of eighteen (n=18) subjects with abnormal glucose tolerance (AGT) were recruited and randomly assigned (2:1 ratio) to receive either 35 1-hour sessions of enhanced external counterpulsation (EECP) with target inflation pressure of 300 mm Hg per cuff (EECP group; n=12) or continued medical care with no EECP intervention (Time-Control group; n=6).

Subject Characteristics before EECP and Time-Control

The baseline characteristics for the EECP and Time-Control subjects are presented in Table 4-1. The EECP and Time-Control groups did not differ with respect to age, body weight, body height, body mass index, body fat percentage, trunk to limb fat mass ratio, metformin therapy, lipid-lowering therapy, beta-blocker therapy, calcium-channel blocker therapy, angiotensin converting enzyme (ACE) inhibitor/angiotensin-II receptor blocker (ARB) therapy, or diuretic therapy. There were no changes in body weight, body mass index, and body fat percentage in either the EECP or Time-Control group following 35 sessions or 7 weeks respectively.

Brachial Artery Endothelial Function after EECP or Time-Control

Brachial artery flow mediated dilation (FMD) results are presented in Table 4-2 and Figures 4-1, 4-2, and 4-3. There was no significant change in baseline brachial artery diameter in the EECP or Time-Control groups. As shown in Figures 4-1 and 4-2 there was a significant increase in brachial artery FMD (2.90 ± 0.31% vs. 4.01 ± 0.59%, \( P < 0.05 \)) and absolute change in diameter (0.13 ± 0.01mm vs. 0.18 ± 0.02mm, \( P < 0.05 \)) in the EECP group following 35 sessions compared to baseline. There was no significant change in brachial artery FMD (3.22 ± 0.34% vs. 3.16 ± 0.22%, \( P = \text{NS} \)) or
absolute change in diameter (0.14 ± 0.01 mm vs. 0.13 ± 0.02 mm, \( P = \text{NS} \)) in the Time-Control group at 7 weeks following baseline. As shown in Figure 4-3, there was a significant increase in the normalized brachial FMD response (0.15 ± 0.02 s\(^{-1}\) vs. 0.19 ± 0.02 s\(^{-1}\), \( P \leq 0.05 \)) in the EECP group following 35 sessions compared to baseline, but there was no significant change in the normalized FMD response (0.16 ± 0.03 s\(^{-1}\) vs. 0.15 ± 0.02 s\(^{-1}\), \( P = \text{NS} \)) in the Time-Control group.

**Popliteal Artery Endothelial Function after EECP or Time-Control**

Popliteal artery FMD results are presented in Table 4-3 and Figures 4-4, 4-5, and 4-6. There was no significant change in baseline popliteal artery diameter in the EECP or Time-Control groups. There was also no significant change in popliteal FMD or absolute change in diameter in the EECP or Time-Control groups following 35 sessions or 7 weeks, respectively. As shown in Figure 4-6, there was a significant increase in the normalized popliteal FMD response (0.23 ± 0.03 s\(^{-1}\) vs. 0.35 ± 0.04 s\(^{-1}\), \( P < 0.05 \)) in the EECP group following 35 sessions compared to baseline, but there was no significant change in the normalized FMD response (0.26 ± 0.05 s\(^{-1}\) vs. 0.26 ± 0.05 s\(^{-1}\), \( P = \text{NS} \)) in the Time-Control group.

**Forearm and Calf Resistance Artery Blood Flow after EECP or Time-Control**

Forearm resistance artery blood flow values during reactive hyperemia are presented in Table 4-4 and Figures 4-7, 4-8, and 4-9. There was no significant change in resting forearm blood flow (FBF) for the EECP or Time-Control groups. Peak FBF significantly increased 26\% (15.68 ± 1.47 mL/min/100mL vs. 19.71 ± 1.56 mL/min/100mL, \( P \leq 0.05 \)) in the EECP group after 35 sessions compared to baseline, but there was no significant change in the Time-Control group (16.49 ± 0.94 mL/min/100mL vs. 16.86 ± 1.12 mL/min/100mL, \( P = \text{NS} \)). Total FBF area under the
curve (AUC$_{3\text{min}}$) increased significantly 37% (5.04 ± 0.63 mL/min/100mL vs. 6.91 ± 0.74 mL/min/100mL, $P \leq 0.05$) in the EECP group after 35 sessions compared to baseline, but there was no significant change in the Time-Control group (5.53 ± 0.88 mL/min/100mL vs. 5.63 ± 0.96 mL/min/100mL, $P = \text{NS}$).

Calf resistance artery blood flow values during reactive hyperemia are presented in Table 4-5 and Figures 4-10, 4-11, and 4-12. There were no significant changes in resting calf blood flow (CBF), peak CBF, or total CBF AUC$_{3\text{min}}$ in the EECP or Time control groups after 35 sessions or 7 weeks respectively, compared to baseline.

**Markers of Angiogenesis/Vasculogenesis after EECP or Time-Control**

Plasma levels of vascular endothelial growth factor (VEGF), mean fiber area, skeletal muscle capillary density, and capillary to fiber ratio values are presented in Table 4-6 and Figures 4-13, 4-14, and 4-15. Additionally, a representative picture of capillary visualization from periodic acid-Schiff's base staining is presented in Figure 4-16. Mean fiber area did not change from baseline in the EECP or Time-Control groups. There was a significant increase in plasma VEGF (9.74 ± 1.59 pg/mL vs. 17.04 ± 2.65 pg/mL, $P \leq 0.05$) in the EECP group after 35 sessions, but no change (9.94 ± 2.24 pg/mL vs. 8.99 ± 3.75 pg/mL, $P = \text{NS}$) in the Time-Control group compared to baseline.

There was no significant difference in skeletal muscle capillary density in either the EECP or Time-Control groups, respective to baseline. However, capillary to fiber ratio was significantly increased (1.71 ± 0.11 vs. 1.84 ± 0.11, $P \leq 0.05$) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (1.65 ± 0.16 vs. 1.63 ± 0.16, $P = \text{NS}$).
Vasoactive Balance after EECP or Time-Control

Plasma levels of nitrite/nitrate (NOx), 6-keto-prostaglandin 1α (6-keto-PGF1α), and endothelin (ET-1) are presented in Table 4-7 and Figures 4-17, 4-18, and 4-19. There was a significant increase in plasma NOx (26.47 ± 1.58 µmol/L vs. 34.46 ± 1.78 µmol/L, \( P < 0.05 \)) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (26.40 ± 2.34 µmol/L vs. 26.87 ± 2.63 µmol/L, \( P = \text{NS} \)). Furthermore, plasma concentrations of NOx following EECP were significantly greater than the Time-Control group at the same time-point (\( P < 0.05 \)). There was a significant increase in plasma levels of 6-keto-PGF1α (112.1 ± 15.3 pg/mL vs. 163.4 ± 11.7 pg/mL, \( P < 0.05 \)) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (130.3 ± 21.6 pg/mL vs. 126.3 ± 16.6 pg/mL, \( P = \text{NS} \)). There was no significant change in plasma ET-1 or the NOx/ET-1 ratio in either group.

Lipid Peroxidation, Antioxidant Capacity, and Endogenous Nitric Oxide Inhibition by ADMA after EECP or Time-Control

Plasma levels of 8-isoprostane-F_{2α} (8-iso-PGF_{2α}), oxygen radical absorbance capacity (ORAC), and asymmetric dimethylarginine (ADMA) are presented in Table 4-8 and Figures 4-20, and 4-21. There was a significant decrease in plasma levels of 8-iso-PGF_{2α} (838.2 ± 61.2 pg/mL vs. 644.0 ± 70.7 pg/mL, \( P < 0.05 \)) in the EECP group following 35 sessions, but no significant change in the Time-Control group (749.9 ± 86.6 pg/mL vs. 811.9 ± 99.9 pg/mL, \( P = \text{NS} \)). There was no significant change in the ORAC of plasma in either group. There was a significant decrease in plasma levels of ADMA (0.47 ± 0.01 µmol/L vs. 0.42 ± 0.01 µmol/L, \( P < 0.05 \)) in the EECP group following 35 sessions, but no significant change in the Time-Control group (0.45 ± 0.02 µmol/L vs. 0.46 ± 0.02 µmol/L, \( P = \text{NS} \)). In addition, plasma concentrations of ADMA following
EECP were significantly less than the Time-Control group at the same time-point \( (P \leq 0.05) \).

**Plasma Markers of Inflammation after EECP or Time-Control**

Plasma levels of high sensitivity C-reactive protein (hsCRP) and tumor necrosis factor-α (TNF-α) are presented in Table 4-9 and Figures 4-22 and 4-23. There was a significant decrease in plasma levels of hsCRP (2.67 ± 0.38 mg/L vs. 1.91 ± 0.33 mg/L, \( P \leq 0.05 \)) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (2.62 ± 0.51 mg/L vs. 2.52 ± 0.44 mg/L, \( P = \text{NS} \)). There was no significant change in TNF-α plasma concentrations in either group.

**Fasting Markers of Glycemic Control after EECP or Time-Control**

Fasting plasma glucose (FPG), fasting plasma insulin (FPI), homeostasis model assessment of insulin resistance (HOMA\(_{\text{IR}}\)), quantitative insulin sensitivity check index (QUICKI), and glycosylated hemoglobin (HbA1c) values at baseline and after 35 sessions of EECP or 7 weeks of Time-Control are presented in Table 4-10 and Figures 4-24, 4-25, 4-26, 4-27, and 4-28. There was a significant decrease in FPG (143.9 ± 8.5 mg/dL vs. 127.0 ± 6.6 mg/dL, \( P \leq 0.05 \)) in the EECP group following 35 sessions, but no significant change in the Time-Control group (138.3 ± 12.0 mg/dL vs. 140.2 ± 9.3 mg/L, \( P = \text{NS} \)). FPI did not change in either the EECP or Time-Control group following 35 sessions or 7 weeks of standard care respectively. The HOMA\(_{\text{IR}}\) was significantly decreased (3.02 ± 0.55 vs. 2.08 ± 0.39, \( P \leq 0.05 \)) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (3.25 ± 0.78 vs. 3.38 ± 0.56, \( P = \text{NS} \)). The QUICKI was significantly increased (0.322 ± 0.009 vs. 0.335 ± 0.009, \( P \leq 0.05 \)) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (0.321 ± 0.013 vs. 0.321 ± 0.013, \( P = \text{NS} \)).
HbA1c did not change in the EECP (6.63 ± 0.29% vs. 6.59 ± 0.26%, \( P = \text{NS} \)) or Time-Control (6.50 ± 0.41% vs. 6.53 ± 0.37%, \( P = \text{NS} \)) groups.

**Dynamic Indicies of Glucose Tolerance Derived from the Oral Glucose Tolerance Test after EECP or Time-Control**

Plasma glucose concentrations 120 minutes after initiation of oral glucose tolerance testing (PPG\(_{120}\)), the oral glucose insulin sensitivity index (OGIS), and the composite whole-body insulin sensitivity index (ISI) at baseline and after EECP or Time-Control are presented in Table 4-11 and figures 4-29, 4-30, and 4-31. There was a significant decrease in PPG\(_{120}\) (224.4 ± 24.6 mg/dL vs. 196.1 ± 24.7 mg/dL, \( P < 0.05 \)) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (246.0 ± 34.8 mg/dL vs. 249 ± 34.9 mg/L, \( P = \text{NS} \)). The OGIS significantly increased (271.9 ± 10.5 mL·min\(^{-1}·m^{-2}\) vs. 311.5 ± 12.3 mL·min\(^{-1}·m^{-2}\), \( P < 0.05 \)) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (287.4 ± 14.8 mL·min\(^{-1}·m^{-2}\) vs. 282.4 ± 17.4 mL·min\(^{-1}·m^{-2}\), \( P = \text{NS} \)). Moreover, The ISI (composite) significantly increased (2.77 ± 0.40 vs. 3.36 ± 0.42, \( P < 0.05 \)) in the EECP group following 35 sessions, but did not change significantly in the Time-Control group (2.69 ± 0.57 vs. 2.50 ± 0.59, \( P = \text{NS} \)).

**Western Blot Analysis of Protein Expression in Vastus Lateralis Skeletal Muscle Biopsy Homogenate after EECP or Time-Control**

Western blot analysis of p-protein kinase B (Akt)\(_{1/2/3}/\text{Akt}_{1/2}\), p-5'-adenosine monophosphate-activated protein kinase (AMPK)\(_{\alpha2}/\text{AMPK}_{\alpha}\), p-TBC1 domain family member 4 (TBC1D4)/TBC1D4, glucose transporter-4 (GLUT-4), endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx), and 4-hydroxynonenal (4-HNE) expression in vastus lateralis skeletal
muscle biopsy homogenate are presented in Table 4-12 and Figures 4-32, 4-33, 4-34, 4-35, 4-36, 4-37, 4-38, 4-39, 4-40, and 4-41. To assess phosphorylation state of Akt, AMPK, and TBC1D4, phosphorylated protein is expressed relative to total protein as a ratio. All other protein expression was normalized to β-actin loading control. p-Akt$_{1/2/3}$/Akt$_{1/2}$, p-AMPK$_{α2}$/AMPK$_{α}$, and p-TBC1D4/TBC1D4 did not change in either the EECP or Time-Control groups. There was a significant increase in expression of GLUT-4 in the EECP group (+47.24 ± 13.92%, $P < 0.05$). There was no change in GLUT-4 expression in the Time-Control group (+3.79 ± 11.64%, $P = NS$). eNOS protein expression increased significantly in the EECP group (+87.32 ± 20.30%, $P < 0.01$), but there was no change in the Time-Control group (+1.53 ± 26.64%, $P = NS$). There was a trend for increased expression of nNOS in the EECP group (+27.39 ± 11.12%, $P < 0.10$), however it did not reach statistical significance. There was no change in nNOS expression in the Time-Control group (-4.05 ± 9.79%, $P = NS$). There was no change in MnSOD expression in either group. There was a trend for increased expression of CuZnSOD in the EECP group (+33.37 ± 11.89%, $P < 0.10$), and there was no change in CuZnSOD expression in the Time-Control group (-2.70 ± 14.55%, $P = NS$). In addition, there was a trend for increased expression of GPx in the EECP group (+25.79 ± 7.06%, $P < 0.10$), however it did not reach statistical significance. There was no change in GPx expression in the Time-Control group (+7.90 ± 9.94%, $P = NS$). Finally, there was no change in 4-HNE, a marker of oxidative modification of proteins, in either group.
Table 4-1. Baseline subject characteristics before enhanced external counterpulsation (EECP) or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>64.31 ± 1.86</td>
<td>64.00 ± 3.03</td>
<td>0.933</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>97.20 ± 4.94</td>
<td>99.22 ± 7.46</td>
<td>0.666</td>
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<tr>
<td>Body height (cm)</td>
<td>178.7 ± 19.8</td>
<td>179.1 ± 18.5</td>
<td>0.893</td>
</tr>
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<td>Body mass index (kg/cm^2)</td>
<td>30.33 ± 1.21</td>
<td>30.83 ± 1.93</td>
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<td>Body fat (%)</td>
<td>32.05 ± 1.71</td>
<td>32.67 ± 2.41</td>
<td>0.628</td>
</tr>
<tr>
<td>Trunk-to-limb fat mass ratio</td>
<td>1.61 ± 0.07</td>
<td>1.71 ± 0.16</td>
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<td>Metformin therapy, no. (%)</td>
<td>3 (25)</td>
<td>1 (17)</td>
<td>0.709</td>
</tr>
<tr>
<td>Sulfonylurea therapy, no. (%)</td>
<td>2 (17)</td>
<td>1 (17)</td>
<td>0.999</td>
</tr>
<tr>
<td>Lipid-lowering therapy, no. (%)</td>
<td>7 (58)</td>
<td>3 (50)</td>
<td>0.755</td>
</tr>
<tr>
<td>Beta-blocker therapy, no. (%)</td>
<td>2 (17)</td>
<td>1 (17)</td>
<td>0.990</td>
</tr>
<tr>
<td>CCB therapy, no. (%)</td>
<td>3 (25)</td>
<td>1 (17)</td>
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</tr>
<tr>
<td>ACE-I/ARB therapy, no. (%)</td>
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<td>2 (33)</td>
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<td>Diuretic therapy, no. (%)</td>
<td>3 (25)</td>
<td>1 (17)</td>
<td>0.709</td>
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Values are mean ± SEM; CCB = calcium channel blocker; ACE-I = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker

Table 4-2. Brachial artery flow-mediated dilation (FMD) at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
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<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
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<tr>
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<td>Baseline</td>
<td>Final</td>
<td>Baseline</td>
</tr>
<tr>
<td>Baseline diameter (mm)</td>
<td>4.49±0.15</td>
<td>4.55±0.13</td>
<td>4.33±0.20</td>
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<tr>
<td>Absolute dilation (mm)</td>
<td>0.13±0.01</td>
<td>0.18±0.02*</td>
<td>0.14±0.01</td>
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<tr>
<td>Brachial FMD (%)</td>
<td>2.90±0.31</td>
<td>4.01±0.59*</td>
<td>3.22±0.34</td>
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<tr>
<td>Normalized FMD (s^{-1})</td>
<td>0.15±0.02</td>
<td>0.19±0.02*</td>
<td>0.16±0.03</td>
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</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group. FMD = flow-mediated dilation

Table 4-3. Popliteal artery flow-mediated dilation (FMD) at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
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<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
<td>Baseline</td>
</tr>
<tr>
<td>Baseline diameter (mm)</td>
<td>6.28±0.39</td>
<td>6.25±0.42</td>
<td>6.49±0.30</td>
</tr>
<tr>
<td>Absolute dilation (mm)</td>
<td>0.09±0.01</td>
<td>0.15±0.02</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>Popliteal FMD (%)</td>
<td>1.50±0.15</td>
<td>2.43±0.24</td>
<td>1.73±0.12</td>
</tr>
<tr>
<td>Normalized FMD (s^{-1})</td>
<td>0.23±0.03</td>
<td>0.35±0.04*</td>
<td>0.26±0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group. FMD = flow-mediated dilation
Table 4-4. Forearm venous occlusion plethysmography parameters at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
</tr>
<tr>
<td>Resting FBF</td>
<td>1.90 ± 0.15</td>
<td>1.86 ± 0.12</td>
</tr>
<tr>
<td>Peak FBF</td>
<td>15.68 ± 1.47</td>
<td>19.71 ± 1.56*</td>
</tr>
<tr>
<td>Total FBF AUC₃min</td>
<td>5.04 ± 0.63</td>
<td>6.91 ± 0.74*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group. Units are mL/min/100mL tissue; FBF = forearm blood flow; AUC = area under flow * time curve

Table 4-5. Calf venous occlusion plethysmography parameters at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
</tr>
<tr>
<td>Resting CBF</td>
<td>1.65 ± 0.19</td>
<td>1.61 ± 0.17</td>
</tr>
<tr>
<td>Peak CBF</td>
<td>16.36 ± 1.77</td>
<td>18.03 ± 1.50</td>
</tr>
<tr>
<td>Total CBF AUC₃min</td>
<td>4.35 ± 0.62</td>
<td>4.94 ± 0.59</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group. Units are mL/min/100mL tissue; CBF = calf blood flow; AUC = area under flow * time curve

Table 4-6. Vascular endothelial growth factor (VEGF) and capillary density parameters at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
</tr>
<tr>
<td>VEGF (pg/mL)</td>
<td>9.74 ± 1.59</td>
<td>17.04 ± 2.65*</td>
</tr>
<tr>
<td>Fiber area (µm²)</td>
<td>4883 ± 514</td>
<td>5390 ± 544</td>
</tr>
<tr>
<td>CD (capillaries/mm²)</td>
<td>341.5 ± 20.7</td>
<td>360.1 ± 21.5</td>
</tr>
<tr>
<td>C/F (ratio)</td>
<td>1.71 ± 0.11</td>
<td>1.84 ± 0.11*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group. VEGF = vascular endothelial growth factor; CD = capillary density; C/F = capillaries per fiber

Table 4-7. Vasoactive balance at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
</tr>
<tr>
<td>NOx (µmol/L)</td>
<td>26.47 ± 1.58</td>
<td>34.46 ± 1.78*</td>
</tr>
<tr>
<td>6-keto-PGF₁α (pg/mL)</td>
<td>112.1 ± 15.3</td>
<td>163.4 ± 11.7*</td>
</tr>
<tr>
<td>ET-1 (pg/mL)</td>
<td>2.54 ± 0.34</td>
<td>2.28 ± 0.32</td>
</tr>
<tr>
<td>NOx/ET-1 (A.U.)</td>
<td>8.89 ± 1.40</td>
<td>12.58 ± 2.05</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group; #P < 0.05 vs. Time-Control at same time-point. NOx = nitrite/nitrate; ET-1 = endothelin-1; 6-keto-PGF₁α = 6-keto prostaglandin F₁α
Table 4-8. Lipid peroxidation, antioxidant capacity, and endogenous nitric oxide (NO) inhibition at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Final</td>
<td>Baseline Final</td>
</tr>
<tr>
<td>8-iso-PGF(_2\alpha) (pg/mL)</td>
<td>838.2 ± 61.2 644.0 ± 70.7*</td>
<td>749.9 ± 86.6 811.9 ± 99.9</td>
</tr>
<tr>
<td>ORAC (µM trolox)</td>
<td>462.2 ± 1.66 465.7 ± 1.19</td>
<td>463.3 ± 2.35 462.8 ± 1.69</td>
</tr>
<tr>
<td>ADMA (µmol/L)</td>
<td>0.47 ± 0.01 0.42 ± 0.01*</td>
<td>0.45 ± 0.02 0.46 ± 0.02</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group; #P < 0.05 vs. Time-Control at same time-point. PGF\(_2\alpha\) = prostaglandin F\(_2\alpha\) isoprostanes; ORAC = oxygen radical absorbance capacity; ADMA = asymmetric dimethylarginine.

Table 4-9. Biomarkers of inflammation at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Final</td>
<td>Baseline Final</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>2.67 ± 0.38 1.91 ± 0.33*</td>
<td>2.62 ± 0.51 2.52 ± 0.44</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>4.10 ± 0.33 3.84 ± 0.36</td>
<td>4.11 ± 0.46 4.15 ± 0.51</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group. hsCRP = high sensitivity C-reactive protein; TNF-α = tumor necrosis factor-α; IL-6 = interleukin-6.

Table 4-10. Fasting markers of glycemic control at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Final</td>
<td>Baseline Final</td>
</tr>
<tr>
<td>FPG (mg/dL)</td>
<td>143.9 ± 8.5 127.0 ± 6.6*</td>
<td>138.3 ± 12.0 140.2 ± 9.3</td>
</tr>
<tr>
<td>FPI (µIU/mL)</td>
<td>10.52 ± 1.45 8.72 ± 1.21</td>
<td>9.71 ± 1.96 9.87 ± 1.63</td>
</tr>
<tr>
<td>HOMA(_{IR}) (A.U.)</td>
<td>3.02 ± 0.55 2.08 ± 0.39*</td>
<td>3.25 ± 0.78 3.38 ± 0.56</td>
</tr>
<tr>
<td>QUICKI (A.U.)</td>
<td>0.322 ± 0.009 0.335 ± 0.009*</td>
<td>0.321 ± 0.013 0.321 ± 0.013</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.63 ± 0.29 6.59 ± 0.26</td>
<td>6.50 ± 0.41 6.53 ± 0.37</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group. FPG = fasting plasma glucose; FPI = fasting plasma insulin; HOMA\(_{IR}\) = homeostatic model assessment – insulin resistance; QUICKI = quantitative insulin sensitivity check index; HbA1c = glycosylated hemoglobin.

Table 4-11. Dynamic indices of glucose tolerance at baseline and after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Final</td>
<td>Baseline Final</td>
</tr>
<tr>
<td>PPG(_{120}) (mg/dL)</td>
<td>224.4 ± 24.6 196.1 ± 24.7*</td>
<td>246.0 ± 34.8 249.4 ± 34.9</td>
</tr>
<tr>
<td>OGIS(_{120})</td>
<td>271.9 ± 10.5 311.5 ± 12.3*</td>
<td>287.4 ± 14.8 282.4 ± 17.4</td>
</tr>
<tr>
<td>ISI (composite)</td>
<td>2.77 ± 0.40 3.36 ± 0.42*</td>
<td>2.69 ± 0.57 2.50 ± 0.59</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. baseline within-group. PPG\(_{120}\) = post-prandial glucose at 120 minutes; OGIS\(_{120}\) = oral glucose insulin sensitivity index (Mari et al. index),\(^\text{65}\) measured in mL·min\(^{-1} \cdot m^{-2}\); ISI (composite) = composite whole-body insulin sensitivity index (Matsuda et al. index),\(^\text{58}\) measured in A.U.
Table 4-12. Percent change in protein expression from vastus lateralis skeletal muscle biopsy homogenate after EECP or Time-Control

<table>
<thead>
<tr>
<th></th>
<th>EECP (n=12)</th>
<th>Time-Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Akt1/2/3 /Akt1/2 (%)</td>
<td>-4.41 ± 12.14</td>
<td>2.42 ± 1.80</td>
</tr>
<tr>
<td>p-AMPKα2/AMPKα (%)</td>
<td>-4.40 ± 7.38</td>
<td>0.17 ± 2.50</td>
</tr>
<tr>
<td>p-TBC1D4/TBC1D4 (%)</td>
<td>2.81 ± 12.40</td>
<td>5.38 ± 7.34</td>
</tr>
<tr>
<td>GLUT-4/β-actin (%)</td>
<td>47.24 ± 13.92*</td>
<td>3.79 ± 11.64</td>
</tr>
<tr>
<td>eNOS/β-actin (%)</td>
<td>87.32 ± 20.30*</td>
<td>1.53 ± 26.64</td>
</tr>
<tr>
<td>nNOS/β-actin (%)</td>
<td>27.39 ± 11.12†</td>
<td>-4.05 ± 9.79</td>
</tr>
<tr>
<td>MnSOD/β-actin (%)</td>
<td>9.59 ± 12.37</td>
<td>19.96 ± 13.33</td>
</tr>
<tr>
<td>CuZnSOD/β-actin (%)</td>
<td>33.37 ± 11.89†</td>
<td>-2.70 ± 14.55</td>
</tr>
<tr>
<td>GPx/β-actin (%)</td>
<td>25.79 ± 7.06†</td>
<td>-4.08 ± 11.05</td>
</tr>
<tr>
<td>4-HNE/β-actin (%)</td>
<td>-12.29 ± 4.76</td>
<td>7.90 ± 9.94</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; *P < 0.05 vs. Time-Control group; †P < 0.10 vs. Time-Control group. Akt = protein kinase B; AMPKα = 5'-adenosine monophosphate-activated protein kinase alpha; TBC1D4 = TBC1 domain family member 4; GLUT-4 = glucose transporter 4; eNOS = endothelial nitric oxide synthase; nNOS = neuronal nitric oxide synthase; MnSOD (SOD-2) = manganese superoxide dismutase; CuZnSOD (SOD-1) = copper-zinc superoxide dismutase; GPx = selenium-dependent cellular glutathione peroxidase.

Figure 4-1. Brachial artery flow-mediated dilation (FMD) at baseline and after enhanced external counterpulsation (EECP) or Time-Control. Data are mean ± SEM. *P < 0.05 vs. baseline within-groups.
Figure 4-2. Brachial artery absolute diameter dilation at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P \leq 0.05$ vs. baseline within-groups.

Figure 4-3. Normalized brachial artery flow-mediated dilation (FMD) at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P \leq 0.05$ vs. baseline within-groups.
Figure 4-4. Popliteal artery flow-mediated dilation (FMD) at baseline and after EECP or Time-Control. Data are mean ± SEM.

Figure 4-5. Popliteal artery absolute diameter dilation at baseline and after EECP or Time-Control. Data are mean ± SEM.
Figure 4-6. Normalized popliteal artery flow-mediated dilation (FMD) at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P < 0.05$ vs. baseline within-groups.

Figure 4-7. Resting forearm blood flow (FBF) at baseline and after EECP or Time-Control. Data are mean ± SEM.
Figure 4-8. Peak forearm blood flow (FBF) at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P < 0.05$ vs. baseline within-groups.

Figure 4-9. Total area under curve (AUC) forearm blood flow (FBF) at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P < 0.05$ vs. baseline within-groups.
Figure 4-10. Resting calf blood flow (CBF) at baseline and after EECP or Time-Control. Data are mean ± SEM.

Figure 4-11. Peak calf blood flow (CBF) at baseline and after EECP or Time-Control. Data are mean ± SEM.
Figure 4-12. Total area under curve (AUC) calf blood flow (CBF) at baseline and after EECP or Time-Control. Data are mean ± SEM.

Figure 4-13. Vascular endothelial growth factor (VEGF) at baseline and after EECP or Time-Control. Data are mean ± SEM. *P < 0.05 vs. baseline within-groups. #P < 0.05 vs. Time-Control group at same time-point.
Figure 4-14. Capillary density (CD) of human vastus lateralis biopsy samples at baseline and after EECP or Time-Control. Data are mean ± SEM.

Figure 4-15. Capillary per fiber ratio of human vastus lateralis biopsy samples at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P < 0.05$ vs. baseline within-groups.
Figure 4-16. Representative photomicrograph of skeletal muscle morphology. Vastus lateralis skeletal muscle biopsies were obtained and histomounts were stained using an amylase-periodic acid Schiff histochemical stain. Fibers and capillaries run perpendicular to the page. The insert bar represents 50 µm. Values of representative photomicrograph: Mean fiber area = 4825 µm$^2$; Capillary Density (CD) = 396 capillaries/mm$^2$; Capillary to Fiber Ratio (C/F) = 1.79.

Figure 4-17. Nitrite/Nitrate (NOx) at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P < 0.05$ vs. baseline within-groups. #$P < 0.05$ vs. Time-Control group at same time-point.
Figure 4-18. Six (6)-keto prostaglandin F1α (6-keto-PGF₁α) at baseline and after EECP or Time-Control. Data are mean ± SEM. *P < 0.05 vs. baseline within-groups.

Figure 4-19. Endothelin-1 (ET-1) at baseline and after EECP or Time-Control. Data are mean ± SEM.
Figure 4-20. Eight (8) iso-prostaglandin-F$_{2\alpha}$ (8-iso-PGF$_{2\alpha}$) at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P < 0.05$ vs. baseline within-groups.

Figure 4-21. Asymmetric dimethylarginine (ADMA) at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P < 0.05$ vs. baseline within-groups. #$P < 0.05$ vs. Time-Control group at same time-point.
Figure 4-22. High sensitivity C-reactive protein (hsCRP) at baseline and after EECP or Time-Control. Data are mean ± SEM. *$P < 0.05$ vs. baseline within-groups.

Figure 4-23. Tumor necrosis factor-α (TNF-α) at baseline and after EECP or Time-Control. Data are mean ± SEM.
Figure 4-24. Fasting plasma glucose (FPG) at baseline and after EECP or Time-Control. Data are mean ± SEM. *P < 0.05 vs. baseline within-groups.

Figure 4-25. Fasting plasma insulin (FPI) at baseline and after EECP or Time-Control. Data are mean ± SEM.
Figure 4-26. Homeostatic model assessment of insulin resistance (HOMA\textsubscript{IR}) at baseline and after EECP or Time-Control. Data are mean ± SEM. *\(P < 0.05\) vs. baseline within-groups.

Figure 4-27. Quantitative insulin sensitivity check index (QUICKI) at baseline and after EECP or Time-Control. Data are mean ± SEM. *\(P < 0.05\) vs. baseline within-groups.
Figure 4-28. Glycosylated hemoglobin (HbA1c) at baseline and after EECP or Time-Control. Data are mean ± SEM.

Figure 4-29. Plasma glucose at 120 minutes after initiation of oral glucose tolerance testing (PPG_{120}) at baseline and after EECP or Time-Control. Data are mean ± SEM. *P < 0.05 vs. baseline within-groups.
Figure 4-30. Oral glucose insulin sensitivity index (OGIS\textsubscript{120}) at baseline and after EECP or Time-Control. Data are mean ± SEM. *\(P < 0.05\) vs. baseline within-groups.

Figure 4-31. Composite whole-body insulin sensitivity index (ISI) at baseline and after EECP or Time-Control. Data are mean ± SEM. *\(P < 0.05\) vs. baseline within-groups.
Figure 4-32. Percent change in phosphorylation state of protein kinase B (p-Akt/Akt) from baseline after EECP and Time-Control. Data are mean percent change ± SEM.

Figure 4-33. Percent change in phosphorylation state of 5'-adenosine monophosphate-activated protein kinase (p-AMPKα/AMPKα) from baseline after EECP and Time-Control. Data are mean percent change ± SEM.
Figure 4-34. Percent change in phosphorylation state of TBC1 domain family member 4 (p-TBC1D4/TBC1D4) from baseline after EECP and Time-Control. Data are mean percent change ± SEM.

Figure 4-35. Percent change in glucose transporter-4 (GLUT-4) protein expression from baseline after EECP and Time-Control. Representative bands from western blot analysis picture. Data are mean percent change ± SEM. * $P < 0.05$ vs. Time-Control group. T1 = baseline time-point; T2 = post time-point; CTL = Time-Control group.
Figure 4-36. Percent change in endothelial nitric oxide synthase (eNOS) protein expression from baseline after EECP and Time-Control. Representative bands from western blot analysis pictured. Presence of band in standard (STD) lane act as positive control for identification of eNOS. Data are mean percent change ± SEM. *$P < 0.05$ vs. Time-Control group. MWM = molecular weight marker; T1 = baseline time-point; T2 = post time-point; CTL = Time-Control group.
Figure 4-37. Percent change in neuronal nitric oxide synthase (nNOS) protein expression from baseline after EECP and Time-Control. Representative bands from western blot analysis pictured. Data are mean percent change ± SEM. †P < 0.10 vs. Time-Control group. T1 = baseline time-point; T2 = post time-point; CTL = Time-Control group.

Figure 4-38. Percent change in manganese superoxide dismutase (MnSOD) protein expression from baseline after EECP and Time-Control. Data are mean percent change ± SEM.
Figure 4-39. Percent change in copper-zinc superoxide dismutase (CuZnSOD) protein expression from baseline after EECP and Time-Control. Representative bands from western blot analysis pictured. Data are mean percent change ± SEM. †P < 0.10 vs. Time-Control group. T1 = baseline time-point; T2 = post time-point; CTL = Time-Control group.
Figure 4-40. Percent change in selenium-dependent cellular glutathione peroxidase (GPx) protein expression from baseline after EECP and Time-Control. Representative bands from western blot analysis pictured. Data are mean percent change ± SEM. †P < 0.10 vs. Time-Control group. T1 = baseline time-point; T2 = post time-point; CTL = Time-Control group.

Figure 4-41. Percent change in 4-hydroxynonenal (4-HNE) adducts from baseline after EECP and Time-Control. Data are mean percent change ± SEM.
To our knowledge, this is the first study to evaluate the effects of enhanced external counterpulsation (EECP) on arterial function, fasting measures of glycemic control, dynamic measures of glucose tolerance, capillary density, and skeletal muscle protein expression in patients with abnormal glucose tolerance (AGT). The main findings of this study are that, in patients with AGT: 1) EECP therapy elicits similar changes in arterial function to those observed in coronary artery disease (CAD) patients; 2) increases the capillary to fiber ratio in skeletal muscle 3) increases endothelial nitric oxide synthase (eNOS) protein expression; 4) increases fasting measures of glycemic control; and 5) increases dynamic measures of glucose tolerance.

**Peripheral Conduit Artery Endothelial Function and EECP**

The present study demonstrated that 35-sessions of EECP in patients with AGT improved brachial artery flow mediated dilation (FMD) 38% from baseline and popliteal artery FMD 62% from baseline. Similarly, brachial and popliteal artery normalized FMD increased 27% and 52%, respectively from baseline. This is in agreement with EECP previous studies in other patient groups. Braith et al. have previously demonstrated in a sham controlled study of CAD patients that brachial and femoral artery FMD increases 51% and 30% respectively. The distal occlusion method of assessing FMD of the peripheral conduit arteries, as used in the present study, has been shown to be a valid and reliable surrogate of nitric oxide (NO)-mediated endothelial function. Although non-normalized popliteal artery FMD was not significant between groups, the normalized FMD response was significantly different (0.23 ± 0.03 vs. 0.35 ± 0.04, \( P \leq \))
The disparity in the two measures is likely due to the small sample size recruited for the present study. Absolute changes in popliteal artery FMD are similar to those observed in the brachial artery despite larger baseline diameters and lesser increases in hyperemic shear stress responses. Hyperemic shear stimulus is greater in small arteries due to the dependence of post-ischemic flow on radius squared.\textsuperscript{159} However, when normalized to the shear stimulus, the popliteal artery exhibits an FMD response at least equal to that of the brachial artery.\textsuperscript{160, 161} Therefore, without significant changes within or between groups in the post-ischemic shear stimulus, as observed in this study, the power to detect significant changes in normalized popliteal artery FMD is greater due to an increase in the magnitude of change and a decrease in variability.

The mechanism responsible for the observed changes in peripheral artery FMD is likely blood flow shear stress. During a bout of EECP therapy, the sequential inflation (~300 mm Hg) of the 3 pneumatic EECP cuffs from calves to buttocks produces a robust retrograde pressure wave in the femoral arteries and simultaneous moderate-pressure antegrade flow in the brachial arteries.\textsuperscript{26, 46} In a porcine EECP model brachial artery blood flow velocity was shown to increase by 132% and brachial artery wall shear stress increased by >200\%\textsuperscript{26} during lower body compression of pneumatic EECP cuffs. It is not unreasonable to conclude that similar alteration of blood flow velocity and shear stress is realized throughout the entire arterial tree, including the popliteal artery. Consideration should also be given to the compression and relaxation of the pneumatic cuffs that occur repeatedly throughout a 60 minute session of EECP therapy. For example, in a patient with a heart rate of 60 bpm there are approximately 3600 cycles of compression and relaxation inducing systolic reactive hyperemia in the legs.
In summary, EECP increases upper and lower body peripheral arterial function, as measured by FMD, following 35-sessions in patients with AGT. The FMD technique is a non-invasive bioassay of NO bioavailability. Therefore, the robust changes in arterial blood flow and shear stress during EECP therapy may mediate changes in FMD response due to an increase in NO production and/or a decrease in NO degradation. Indeed, in the present study, markers of oxidative stress were depressed, markers of NO plasma concentrations were improved, and inhibitors of NO were depressed. These alterations in NO bioavailability are discussed in greater detail later in this chapter.

**Peripheral Resistance Artery Endothelial Function and EECP**

Our results are similar to previous studies that have demonstrated improvements in peak forearm blood flow (FBF) and calf blood flow (CBF) with reactive hyperemia. We observed a 26% increase in peak FBF and a 10% increase in peak CBF following 35-sessions of EECP in subjects with AGT. This increase in the maximal dilatory capacity in response to reactive hyperemia provides support for our hypothesis that the repetitive inflation and deflation cycles of cuff compression, and resulting alterations in blood flow velocity and shear stress during EECP therapy, improves endothelial function in the peripheral resistance arteries.

Increased post-occlusion blood flow suggests increased capillary or arteriolar proliferation, or improved resistance vessel endothelial function. In the present study, the peripheral conduit artery FMD results support the potential for an improvement in endothelial function. EECP has been shown to acutely increase blood flow in various vascular beds which translates to enhanced shear stress, a key factor in endothelial function. A major contributor to the maximal vasodilatory capacity of the peripheral
resistance arteries is nitric oxide bioavailability. As discussed in the previous section, EECP manifested changes indicative of increased potential for NO production and decreased inhibition of its production. However, the potential effects of angiogenesis/vasculogenesis after EECP and the maximal vasodilatory response must be considered, as they cannot be directly measured using the non-invasive venous occlusion plethysmography (VOP) technique employed in the present study.

**Angiogenesis/Vasculogenesis and EECP**

Plasma levels of vascular endothelial growth factor (VEGF) were elevated to 175% of baseline levels following 35-sessions of EECP in subjects with AGT. VEGF is a potent mediator of both angiogenesis and vasculogenesis that is released in response to hypoxia. In this model of EECP, it is plausible that several thousand brief, intermittent bouts of hypoxia are induced by the high pressure compression cuffs. During a single 60 minute session of EECP therapy in a subject with an average heart rate of 60 bpm, approximately 3600 high pressure compressions are realized. VEGF can also be produced in response to circulating cytokines which may be acutely elevated during a bout of EECP, similar to an exercise response. Despite VEGF not being the only promoter of angiogenesis, basic fibroblast growth factor (FGF2) and hepatocyte growth factor (HGF) have also been implicated, the near two-fold increase in VEGF observed following EECP supports a role for angiogenesis/vasculogenesis in the adaptive mechanisms to EECP therapy.

Shear stress has also been proposed as a major physiological signal in the stimulation of capillary growth. Hoier et al. have demonstrated that two weeks of passive leg movement training, with negligible vastus lateralis electromyography (EMG) activity, induced significant increases in interstitial VEGF, endothelial nitric oxide
synthase (eNOS) messenger ribonucleic acid (mRNA), and capillary to fiber ratios. In that study, blood flow was increased to about 3-times baseline values during training, similar to changes observed with EECP. In the present study, we observed a significant, 8% increase in the number of capillaries per muscle fiber in biopsy sections of the vastus lateralis following 35-sessions of EECP. Although not statistically significant, there was also a trend for increased capillarity density following EECP therapy. The lack of statistical significance may be due to variation fiber area, an effect termed capillary dilution. In the present study, a non-significant increase in mean fiber area was observed, and the analysis of samples using a ratio of capillaries to fibers likely decreased the variability observed in capillary density measures.

Muscle analysis was performed on one small muscle sample from only one skeletal muscle. Although one can speculate on global changes in capillarity, only a site under direct compression was analyzed. However, increased eNOS expression has been demonstrated in tissue upstream from intermittent mechanical compression in rats. Variation due to the fiber type distribution amongst the samples analyzed could also present an additional source of variation, and in the present study, capillarity was not normalized to fiber type. Despite these limitations, efforts were made to achieve the least heterogeneity amongst samples as biopsy sections were taken within 1-2 cm of the baseline site and at the same recorded depth for each procedure. Furthermore, an average of 166 ± 13 fibers were analyzed per sample by selection of sections of tissue with little artifact.

**Vasoactive Balance and EECP**

The present study demonstrated that EECP resulted in a 30% increase in plasma nitrite and nitrate (NOx) levels. Braith et al. have previously demonstrated that plasma
NOx concentrations are elevated 36% following EECP therapy in CAD patients.\textsuperscript{27} Although the increase observed in patients with AGT following 35-sessions of EECP is slightly less in magnitude, this is likely due to greater baseline plasma NOx concentrations in patients with AGT, but without CAD. Increases in NOx as a result of EECP therapy have been reported in both the short and the long term. Akhtar et al. have shown that NOx is increased in the first hour following a single bout of EECP and increases progressively as the course of treatment progresses.\textsuperscript{29} Importantly, the elevated plasma levels are maintained for at least one month following the culmination of 35-sessions of EECP.\textsuperscript{28}

The increase in plasma NOx observed in the present study could be due, in part, to an increase in eNOS and/or neuronal nitric oxide synthase (nNOS) protein expression. We observed a highly significant ($P < 0.01$) 87% increase in eNOS protein expression from baseline in vastus lateralis skeletal muscle biopsy homogenate following 35-sessions of EECP therapy. In addition, there was a trend ($P = 0.067$) for increased nNOS protein expression as protein concentration increased 27% from baseline after 35-sessions of EECP. The increase in nitric oxide synthase (NOS) protein expression with compressive therapy is supported by the findings of Tan et al. who demonstrated that following a single 1 hour bout of intermittent pneumatic compression (IPC) on the hind limbs of Sprague Dawley rats, eNOS and nNOS protein expression was upregulated 120% and 30% from baseline, respectively.\textsuperscript{25} Importantly, increases in NOS expression were also observed in the upstream cremaster muscles flowing 1-hour of intermittent pneumatic compression.\textsuperscript{25}
NOx is influenced by exogenous sources such as food, saliva formation, gastrointestinal microorganisms, and cigarette smoking and is only a gross index of endothelial NO formation in vivo. However, since all of the subjects in the present study were non-smokers and followed a strict National Institutes of Health (NIH) low nitrate diet guideline for 48 hours prior to their lab visits, we are confident that plasma NOx levels were influenced minimally by exogenous sources. Therefore, the improvements in plasma levels of NOx with concomitant increases in eNOS and nNOS protein expression following 35-sessions of EECP therapy likely reflect an improvement in basal endothelial NO production.

Further evidence for an increase in eNOS protein expression and/or activity is demonstrated by the 46% increase in 6-keto-prostaglanin 1α (6-keto-PGF$_{1α}$), the stable metabolite of prostacyclin (PGI$_2$), following 35-sessions of EECP therapy. Prostaglandins are a family of eicosanoids derived from endothelial cells and shear stress has been shown to be a major stimulus for prostacyclin production from endothelial cells. These results are similar to those previously described by Braith et al. who showed a 71% increase in 6-keto-PGF$_{1α}$ following 35-sessions of EECP in patients with CAD.

Although previous studies have demonstrated a decrease in endothelin-1 (ET-1) with EECP therapy, we did not observe a significant decrease in ET-1 after 35-sessions of EECP in patients with AGT. This could be due to lower basal production of ET-1 at baseline compared to the populations examined in prior studies. Also, the value of ET-1 measures in human plasma is controversial. ET-1 acts in a paracrine fashion
on nearby smooth muscle cells on the adventitia side of the vessel. Therefore, the relevance of circulating ET-1 measured in the plasma remains in question.

**Redox Balance and EECP**

The present study demonstrated that EECP resulted in a 23% decrease in the F2-isoprostan e 8-iso-prostaglandin 2α (8-iso-PGF$_{2α}$) following 35-sessions of EECP in subjects with AGT. This reduction is similar to that observed in CAD patients following EECP therapy,$^{27}$ although the baseline plasma concentrations of 8-iso-PGF$_{2α}$ observed in the present study were lesser in magnitude. 8-iso-PGF$_{2α}$, a prostaglandin like compound that is produced by free-radical mediated lipid peroxidation of arachadonic acid, has been suggested to be the most valid plasma marker to assess oxidative stress in human plasma.$^{166-168}$

Increased oxidative stress is an established contributor to the development and progression of diabetes.$^{95}$ Both type I and type II diabetes are associated with increased production of free radicals and/or impaired antioxidant defenses, shifting redox balance toward greater oxidative stress.$^{96, 97}$ Hyperglycemia has also been shown to promote free radical generation through glucose autotoxidation$^{98}$ and lipid peroxidation of low density lipoprotein (LDL) in a superoxide-dependent pathway.$^{99}$ Free radical production due to hyperglycemia can also occur as glucose interacts with proteins ultimately resulting in the formation of advanced glycation endproducts (AGEs). These AGEs can promote free radical formation and quench and block anti-proliferative effects of nitric oxide through their binding with specific AGEs receptors. Davy et al. have shown that glycemic control correlated well with, 8-iso-PGF$_{2α}$.$^{100}$ Oxidative stress may play an important role in not only vascular function, but also glycemic control as bioavailability of NO may mediate/alter the NO-mediated glucose uptake pathway.
Evidence suggests that asymmetric dimethylarginine (ADMA) is associated with endothelial dysfunction in a number of disorders, including, but not limited to, dyslipidemia, hyperhomocysteinemia, hypertension, CAD, heart failure, renal dysfunction, and Type II Diabetes Mellitus (T2DM).\textsuperscript{112, 113} This is the first study to examine the effects of EECP on circulating levels of ADMA in patients with AGT. We observed a 26\% reduction in plasma ADMA concentrations following 35- sessions of EECP. This finding is similar to what has been reported following EECP therapy in CAD patients\textsuperscript{27} and in Type I Diabetics following exercise training.\textsuperscript{169} ADMA, like NO, is derived from the amino acid L-arginine. The production of ADMA from L-arginine can decrease NO production due to substrate utilization. L-arginine concentration may be even more important for glucose clearance as McConnel et al. have shown that L-arginine infusion during cycling exercise increases glucose uptake in a NO dependent manner.\textsuperscript{114} Since type II diabetics are even more reliant on nitric oxide mediated glucose uptake,\textsuperscript{20, 23} this may be of significant physiological relevance.

Following formation, free ADMA is released into the plasma where it can inhibit NOS and decrease NO bioavailability leading to the development of endothelial dysfunction.\textsuperscript{117} Flow mediated dilation, the non-invasive bioassay of NO bioavailability, also demonstrates a negative linear relationship with ADMA concentrations.\textsuperscript{116} Furthermore, inhibition of NOS impairs microvascular recruitment and blunts insulin stimulated glucose uptake.\textsuperscript{118} Steinberg et al. have shown that insulin resistance was associated with blunted endothelium-dependent vasodilatation, but not endothelium-independent vasodilatation, during intrafemoral artery infusions of sodium nitroprusside and metacholine chloride under euglycemic hyperinsulinemic conditions.\textsuperscript{119}
Oxidative stress appears to be a key modulator of ADMA levels. Oxidative stress is associated with an increase in protein-arginine-N-methyltrasferase family of enzymes (PRMT) activity and gene expression in the cell leading to an increase in ADMA formation. Tetrahydrabiopterin (BH$_4$), a cofactor for NOS, and dimethylargininase (DDAH), the major eliminator of ADMA, are also redox sensitive. Although BH$_4$ and DDAH were not measured in the present study, compelling data from the decrease in 8-iso-PGF$_{2\alpha}$ following 35-sessions of EECP therapy in subjects with AGT suggest a decrease in oxidative stress and subsequently, possibly decreased oxidation of BH$_4$ and DDAH. Osanai et al. showed that ADMA levels in human endothelial cells in vitro decreased with 15 dynes/cm$^2$ of shear stress and DDAH activity increased at shear stress levels of $\geq 25$ dynes/cm$^2$. It is not unreasonable to conclude that DDAH activity may be increased with the magnitude of shear stress invoked with EECP ($>200\%$ of baseline, 49 vs. 23 dynes/cm$^2$ in a porcine model). We also observed a trend ($P < 0.10$) for increased expression of endogenous antioxidant capacity with increases in copper-zinc superoxide dismutase (CuZnSOD) and glutathione peroxidase (GPx). The most common antioxidant deficiencies reported in diabetics are lower levels of vitamin C, GPx and SOD. While the role of antioxidant defense in the treatment and pathology of glycemic control remains controversial, treatment of type T2DM patients with vitamin C or vitamin E decreased glycocelated hemoglobin (HbA1c) levels, improved insulin action, decreased plasma insulin concentrations, and decreased indicators of oxidative stress.

**Inflammation and EECP**

Epidemiological evidence for an association between Type II diabetes and inflammation goes back as far as the 1950s. Increased levels of markers and mediators
of inflammation such as high sensitivity C-reactive protein (hsCRP) and tumor necrosis factor-α (TNF-α) correlate well with incident T2DM. In addition, TNF-α has been shown to cause insulin resistance in experimental models. In the present study, following EECP therapy in patients with AGT, significant reductions in plasma levels of hsCRP (28%) were observed. These results are similar to those observed by Braith and colleagues following 35-sessions of EECP in patients with CAD. Although previous studies of EECP in patients with CAD have demonstrated decreases in plasma TNF-α, no significant change was detected in the present study (-6%). Although baseline TNF-α values were considerably less than this comparable study, the concentrations observed in the present study were approximated as the 90th percentile of healthy control values. Increased bioavailability of NO after EECP therapy is the likely mechanism responsible for the reduction in plasma inflammatory markers. NO serves an anti-inflammatory role by inhibiting the expression of monocyte chemotactic protein-1 (MCP-1) and reducing vascular cell adhesion protein-1 (VCAM-1) expression.

**Fasting Glycemic Control and EECP**

To the best of our knowledge, this is the first study to evaluate the changes in fasting glycemic control following EECP therapy. In the present study, following 35-sessions of EECP, subjects with AGT demonstrated marked improvements in fasting indices of glycemic control. Fasting plasma glucose (FPG) values were decreased nearly 17 mg/dl following EECP (143.9 ± 8.5 mg/dL vs. 127.0 ± 6.6 mg/dL). This 13.3% decline in FPG is similar to that observed with resistance training intervention in older men with T2DM, and aerobic exercise training older subjects with non-insulin dependent Diabetes Mellitus (NIDDM) and impaired glucose tolerance (IGT).
Importantly, these changes occurred in the absence of any lifestyle modification and subject weight, body mass index (BMI), body fat percentage, and measures of central adiposity did not change following EECP therapy. In a large scale population study, each 13 mg/dL increase in FPG is associated with a 13% increase in the relative risk for cardiovascular disease. Furthermore, data from the Framingham Heart Study has demonstrated that for every 10 mg/dL increase in FPG there is an 18% increase in all-cause mortality.

Although simple measures of fasting plasma glucose provide an indication of glycemic control, the inclusion of fasting plasma insulin concentrations allows for a more accurate measure of insulin sensitivity. We observed a 31% decline in homeostasis model assessment of insulin resistance (HOMA\textsubscript{IR}) values and a significant increase in the quantitative insulin sensitivity check index (QUICKI). The HOMA\textsubscript{IR} and QUICKI both correlate well with the glucose disposal rate derived from hyperinsulinemic euglycemic clamp (HEC), the gold standard for measuring peripheral insulin sensitivity. Among the criticisms of the HOMA\textsubscript{IR} are a large coefficient of variation and the pulsatile nature of insulin secretion. However, three separate samples, five minutes apart, of plasma insulin concentration were measured to determine a true average fasting plasma insulin concentration. The mathematical difference between the QUICKI and the HOMA\textsubscript{IR} is simply that the former uses the reciprocal of the logarithm of both glucose and insulin to account for the skewed distribution of fasting insulin values and some argue that the QUICKI may be applied to wider ranges of insulin sensitivity. However, in the present study, significant improvements in both measures were observed.
Despite declines in fasting plasma glucose and indices of fasting glycemic control, there was not a significant decline in HbA1c values following 35-sessions of EECP in subjects with AGT. However, the half life of a red blood cell is about 120 days and reflects the average blood glucose control level during the preceding two to three months. Therefore, with a seven week EECP intervention, the study design may not have allowed us to capture this variable. Further studies should be undertaken to determine the efficacy of EECP therapy for glycemic control as measured with measures of HbA1c at multiple time-points beyond the completion of the standard 35 1-hour sessions of EECP therapy.

**Dynamic Measures of Glucose Tolerance and EECP**

Stronger surrogates of insulin sensitivity can be derived from the multiple sampling of plasma insulin and glucose during an oral glucose tolerance test (OGTT). Following 35-sessions of EECP we observed a 28 mg/dL decrease (224.4 ± 24.6 mg/dL vs. 196.1 ± 24.7 mg/dL) in plasma glucose at 120 minutes (12.6% decline) following the ingestion of a 75 gram sugar water beverage (PPG\textsubscript{120}). PPG\textsubscript{120} from the OGTT is frequently used by physicians as a test for T2DM or IGT. In this context, the EECP group, on average, moved from a response that was consistent with T2DM to that observed in IGT. While we did observe a significant decline in PPG\textsubscript{120}, the effect was modest when compared to the declines in PPG\textsubscript{120} following exercise interventions.\textsuperscript{175-177}

Although there are a plethora of indices based on the OGTT that can be used to estimate insulin sensitivity, for the present study, two were chosen; the oral glucose sensitivity index (OGIS\textsubscript{120}) developed by Mari et al.\textsuperscript{65} and the composite whole-body insulin sensitivity index (ISI composite) developed by Matsuda et al.\textsuperscript{58} The OGIS\textsubscript{120} incorporates glucose load, weight, and body surface area while also highlighting the
change in plasma glucose during the final 30 minutes of the OGTT relative to insulin concentration, much like the hyperinsulinemic euglycemic clamp. The ISI (composite) is a more commonly used surrogate of insulin sensitivity that incorporates fasting and mean plasma glucose and insulin concentrations prior to and during the OGTT. Both indices were chosen due to their high correlation with the HEC (r = 0.73 for both) and because of their validation in a wide variety of lean and obese subjects with AGT.\textsuperscript{58, 59, 65}

In the present study, we observed 15% and 21% increases in the OGIS\textsubscript{120} and ISI (composite) respectively. Given the strong correlation of these indices with the HEC, the results from the present study suggest improvements in dynamic measures of glycemic control.

An inherent limitation to indices of insulin sensitivity derived from the OGTT is that it is impossible to differentiate between whole-body, peripheral, or hepatic insulin sensitivity. However, since this method of glucose ingestion is the most physiologically relevant, improvement in indices of insulin sensitivity from the OGTT are encouraging signs of improved glucose tolerance in subjects with AGT following 35-sessions of EECP therapy.

**Potential Mechanisms for Improvement in Fasting Glycemic Control and Dynamic Indices of Glucose Tolerance: Evidence for the Nitric Oxide Pathway?**

In the present study, we did not observe any changes in the phosphorylation states of Akt, AMPK\textsubscript{\alpha}, or TBC1D4. Therefore, changes in glucose homeostasis may occur independent of changes in skeletal muscle signaling. Given the increasing evidence that NO bioavailability (and redox balance) contributes to glucose handling, the significant increases in NOx and significant decreases in 8-iso-PGF\textsubscript{2\alpha} and ADMA may provide evidence for the nitric oxide-mediated glucose handling. Indeed, fasting
plasma NO concentrations are markedly declined with advancing glucose intolerance. Sodium nitroprusside (SNP), a NO donor, significantly increases glucose uptake in the presence of wortmannin, a phosphoinositide 3-kinase (PI3K) inhibitor, indicating that it is not a PI3K dependent mechanism. In addition, L-NG Nitroarginine methyl ester (L-NAME), a NOS inhibitor, administration does not abolish contraction mediated glucose uptake in skeletal muscle. This suggests that the effects of NO are independent of both insulin and contraction and appear to augment glucose uptake systemically.

Although we hypothesized that NO mediated glucose uptake may be realized through alterations in AMPK signaling, several other mechanism exist by which NO may mediate glucose uptake. In resting muscle, NO stimulates glucose uptake through a cyclic guanosine monophosphate (cGMP)-dependent pathway that may involve cyclic GMP-dependent kinase/protein kinase G (PKG) activation. NO can also directly activate G protein α subunits, specifically the proto-oncogene p21ras for glucose transport, which increases glucose uptake through nuclear factor κB (NFκB) signaling. NFκB activity is increased transiently from treatment with NO donors and reacts rapidly to increase the activity of the extracellular signal-related kinases (ERK), c-Jun-NH₂ terminal kinase (JNK), and p38 subgroups of the mitogen-activated protein kinase (MAPK) family. However, the exact mechanism by which the MAPK subfamily elicits glucose uptake has not been elucidated. In addition to the acute effects of p38MAPK on glucose uptake, p38MAPK may also play a role in the chronic glycemic control as it phosphorylates the transcription factor myocyte enhancer factor-2 (MEF2), which is implicated in the expression of glucose transporter-4 (GLUT-4) protein synthesis from
the peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) gene in skeletal muscle. Although we were unable to quantify PGC-1α expression, we did observe a 47% in GLUT-4 protein expression following 35-sessions of EECP in subjects with AGT.

It is important to note that while we did not observe any changes in the Akt, AMPK and TBC1D4 skeletal muscle cell signaling pathways, we were only able to evaluate one time-point. In the present study, muscle biopsies were performed at 48-72 hours following the last session of EECP therapy in an effort to 'capture' the chronic effect of 35-sessions of EECP therapy in subjects with AGT. Transient changes in signaling pathways could occur acutely with each bout of EECP, similar to the effects observed with a bout of exercise. Therefore, it is possible that we were able to capture increases in total protein content, but not necessarily the signal(s) responsible for the increase in transcription and/or translation.

Reduced nutrient exposure to the metabolizing tissue may also play a major role in the continuum of AGT. Insulin receptors are present throughout the vascular tree and act as vasodilators to increase perfusion and delivery of substrates. The adaptations to 35-sessions of EECP included increases in both peripheral conduit and resistance artery function in patients with AGT. Furthermore, capillary density, like nitric oxide bioavailability, decreases progressively with advancing glucose intolerance. The changes observed in capillary to fiber ratio and endothelial function observed in the present study would suggest that improvements in glucose homeostasis may be realized through increase in vascular reactivity to insulin and nutrient delivery.
Conclusions

AGT is associated with several perturbations in normal human physiology, including, but not limited to, endothelial dysfunction, oxidative stress, depressed nitric oxide bioavailability, progressive declines in capillary density, and inflammation. In the present study, we observed significant improvements in fasting measures of glycemic control and significant improvements in dynamic measures of glucose tolerance and insulin sensitivity. We also observed improvements in measures of endothelial function, nitric oxide bioavailability, and capillary to fiber ratios with concurrent decreases in measures of lipid peroxidation and competitive inhibition of NOS. This study provides novel evidence for the improvement of vascular function and glycemic control in subjects with AGT following EECP therapy.

The multifaceted nature of vascular function and glycemic control makes it difficult to isolate a single mechanism responsible for these adaptations. However, increasing evidence indicates that decreasing nitric oxide bioavailability contributes to the pathology of AGT and the progression to T2DM. Moreover, it has been well characterized that the insulin stimulated pathway of glucose uptake is impaired with AGT. Therefore, of particular significance is the increased reliance on NO-stimulated glucose uptake in patients with AGT and the potential to specifically target this pathway for glycemic control intervention. The improvements in NO bioavailability and endothelial function observed in the present study, as well as capillary to fiber ratios, likely mediate greater delivery of nutrients to ‘nutritive’ tissues (i.e. skeletal muscle) during glycemic challenge. Additionally, NO signaling been has implicated in regulation of GLUT-4 translocation. Although we did not observe changes in AMPK signaling in the present study, NO may contribute to cellular signaling and GLUT-4
translocation via several pathways, including cyclic guanosine monophosphate (cGMP). Thus, the potential for increased NO-mediated translocation of GLUT-4 to the cell membrane during glycemic challenge cannot be ruled out. Further studies should be conducted with multiple muscle biopsy sampling during a HEC to accurately describe cellular signaling associated with glucose uptake. Furthermore, the acute effect of EECP and the impact on skeletal muscle cell signaling should be characterized.


BIOGRAPHICAL SKETCH

Jeffrey Steven Martin is the son of Deborah Anne Martin and Paul Wayne Martin, Jr., the brother of Shane Michael Martin, and the husband of Allison Marie Martin. He was born in Attleboro, Massachusetts and was raised in several states including, but not limited to, Maine, Ohio, and Pennsylvania. Jeffrey graduated from Saint Mark's High School in Wilmington, Delaware and went to the University of Pittsburgh in Pittsburgh, Pennsylvania to pursue his bachelor’s degree. He received his Bachelor of Science degree in movement science with a concentration in exercise science in the year 2004 from the University of Pittsburgh. Following undergraduate school, Jeffrey went to Northeastern University in Boston, Massachusetts to work as a graduate teaching assistant and to pursue his Master of Science degree. Jeffrey received his master’s degree in Clinical Exercise Physiology from Northeastern University in 2006. Following graduate school, Jeffrey began his work as a doctoral student in Dr. Randy W. Braith’s cardiovascular lab at the University of Florida in Gainesville, FL. As a doctoral student, he taught the undergraduate Clinical Exercise Physiology course in addition to academic coursework and research endeavors. Jeffrey received his Ph.D. from the University of Florida in the summer of 2011.