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Exogenous Thyroxine Improves Glucose Intolerance in Insulin Resistant Rats Independent of Static Changes in Insulin Signaling

A thesis submitted in partial satisfaction of the requirements
for the degree

Master of Science

in

Quantitative and Systems Biology

by

Guillermo Vazquez-Anaya

Committee in charge:

Professor Rudy M. Ortiz, Chair
Professor Nestor Oviedo
Professor Fabian Filipp

2016

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The thesis of Guillermo Vazquez-Anaya is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2016

This thesis is dedicated to my wife, friends,
and family for their love and support

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List of Abbreviations

$\Sigma\Delta_{BM,}$ Cumulative change in body mass
AMPK, AMP-activated protein kinase
BM, Body mass
DI1, Deiodinase enzyme type 1
DI2, Deiodinase enzyme type 2
DI3, Deiodinase enzyme type 3
fT4, Free thyroxine
FFA, Free fatty acids
GLUT4, Glucose transporter type 4
IR, Insulin resistance
IRI, Insulin resistance index
MCT, monocarboxylate transporter
Na^{+}-K^{+} ATPase, sodium, potassium- ATPase
NEFA, Non-esterified fatty acids
OLETF, Otsuka Long Evans Tokushima Fatty
oGTT, Oral glucose tolerance test
p-AMPK, phosphorylated AMP-activated protein kinase
SBP, Systolic blood pressure
SE, Standard error
SIRT1, Sirtuin 1
TH, Thyroid hormones
TRs, Thyroid hormone receptors
T2DM, Type 2 diabetes mellitus
T3, Triiodothyronine
T4, Thyroxine
THrβ1- Thyroid Hormone Receptor beta 1
TG, Triglyceride
TSH, Thyroid-stimulating hormone
UCP2- uncoupling protein 2

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Manuscripts in Preparation

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Published Abstracts

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Field of Study

Major Field: Quantitative and Systems Biology

Studies in Physiology
Professor Rudy M. Ortiz

Abstract

Exogenous Thyroxine Improves Glucose Intolerance in Insulin Resistant Rats Independent of Static Changes in Insulin Signaling

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2016
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Both hypothyroidism and hyperthyroidism are associated with glucose intolerance, calling into question the contribution of thyroid hormones (TH) on glucose regulation. TH analogues and derivatives may be effective treatment options for glucose intolerance and insulin resistance (IR), but their potential gluco-regulatory effects during conditions of impaired metabolism are not well described. To assess the effects of thyroxine (T4) on glucose intolerance in a model of insulin resistance, an oral glucose tolerance test (oGTT) was performed on three groups of rats (n=8): 1) lean, Long Evans Tokushima Otsuka (LETO), 2) obese, Otsuka Long Evans Tokushima Fatty (OLETF), and 3) OLETF + T4 (8.0 µg/100g BM/d x 5wks). T4 attenuated glucose intolerance by 15 % and decreased IR index (IRI) by 34% in T4-treated OLETF compared to untreated OLETF despite a 31% decrease in muscle GLUT4 mRNA expression. T4 increased the mRNA expressions of muscle monocarboxylate transporter 10 (MCT10), deiodinase type 2 (DI2), sirtuin 1 (SIRT1), and uncoupling protein 2 (UCP2) by 1.8-, 2.2-, 2.7-, and 1.4-fold, respectively, compared to OLETF. Activation of AMP-activated protein kinase (AMPK) and insulin receptor were not significantly altered suggesting that the improvements in glucose intolerance and IR were independent of enhanced insulin-mediated signaling. The results suggest that T4 treatment increased the influx of T4 in skeletal muscle and, with an increase of DI2, increased the availability of the biologically active T3 to up regulate key factors such SIRT1 and UCP2 involved in cellular metabolism and glucose homeostasis.

Introduction

Thyroid Hormones

Although it has been over a century since the primary thyroid hormone, thyroxine (T4), was isolated much of our understanding of its physiology and biology has been elucidated in the past decades (Klieverik 2009). Thyroid hormones (TH) have a multitude of physiological effects related to thermogenesis, metabolism, heart rate, and body composition (Lin & Sun 2011, Araujo *et al.* 2008). The thyroid gland produces a small amount of T3, triiodothyronine, with T4 comprising its main product, whose syntheses are regulated through a negative feedback system. Circulating levels of TH will regulate the expression of thyrotropin-releasing hormone (TRH) and thyroid stimulating hormone (TSH), which upon binding its receptor will influence iodine uptake, and in turn synthesis of TH (Brent 2012, Klieverik 2009).

T4 has limited affinity to the nuclear thyroid hormone receptors (TRs), with T3 being the most biologically active form of the hormone. The deiodination of T4 to T3 is accomplished by deiodinase enzymes type 1 and 2 (DI1 and DI2), which are responsible of removing an outer ring iodine. Removal of the inner ring iodine is achieved by deiodinase enzyme type 3 (DI3) and/or DI1 producing a biologically inactive form, reverse T3 (rT3) (Bianco *et al.* 2002). The TRs include THrα and THrβ, and their expression distribution varies among the tissues (Brent 2012). Upon the binding of T3 to its nuclear receptors, and with interactions with multiple co-factors, this complex is able to bind the thyroid hormone response element (TRE) and influence transcription (Klievereik 2009). Recently a few TH-specific transporters have been identified. The monocarboxylate transporters, MCT8 and MCT10, are specific for THs and facilitate their transport in and out of the cell (Müller *et al.* 2014). Specifically, MCT10 has wide tissue distribution and can rapidly transport T4, potentiating non-genomic effects (Van Der Deure *et al.* 2010).

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is metabolic disorder that affects millions of people worldwide and its prevalence is expected to increase (Chen *et al.* 2012). Characterized by increased levels of blood glucose due to impairment in insulin potential and/or insulin secretion, multiple tissues and organs are affected. The pathogenesis for T2DM is not fully understood, but it's believed that T2DM may result from defectes in insulin-mediated glucose uptake in muscle, dysfunction of the pancreatic β-cells, disruption of secretory function of adipocytes, and impaired insulin action in the liver. Although there is a genetic predisposition for T2DM a sedentary lifestyle, obesity, and poor diet are principal contributors (King 2012, Lin Y & Sun Z 2010).

Insulin is the primary hormone involved in glucose homeostasis. Normally insulin will bind its cell membrane receptor and trigger a signaling cascade that activates the insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase (PI3K), and AKT ultimately facilitating the translocation of glucose transporter 4 (GLUT4) to the plasma membrane to promote the cellular uptake of circulating glucose (Lin Y & Sun Z 2010). Insulin resistance occurs when insulin can no longer potentiate its signaling cascade and

is generally associated with post-receptor defects. To compensate for reduced sensitivity of insulin the pancreas will produce more insulin until it can no longer produce enough to elicit a tissue response and ultimately resulting in hyperglycemia (Lin Y & Sun Z 2010, Viscarra *et al.* 2011).

With skeletal muscle accounting for about 40-50% of the total body mass, it's the largest contributor to resting energy expenditure and insulin-induced glucose utilization (Marsili *et al.* 2010). Thus, defects in insulin signaling in skeletal muscle are of primary interest to understanding T2DM effects. Insulin-stimulated glucose transport is reduced in skeletal muscle in individuals with T2DM (Lin Y & Sun Z 2010). Along with regulating glucose homeostasis insulin is also responsible for inhibiting lipolytic activity in fat cells (breaking down triglycerides into free fatty acids and glycerol) (Morimoto *et al.* 1998). In skeletal muscle the oxidation of free fatty acids (FFA) provides an alternate source to glucose for energy during shifts in metabolism. The development of insulin resistance is due to the insensitivity of adipocytes to insulin resulting in elevated fatty acids ultimatley leading to a reduction of insulin-stimulated glucose uptake, likely associated with increased lipid deposition. This is characteristic of obese subjects with T2DM that display a reduction in oxidation of fat and increased levels of circulating FFA. Obesity, a major risk factor of T2DM, is believed to increase triglycerides and suppress homrones that contribute to insulin sensitivity (Hussain & Hydrie 2010). The inability of insulin to regulate the use of either FFA oxidation or glucose oxidation are key components of T2DM. However, the cause of these impairments in muscle are not fully understood and remain to be elucidated (Hussain & Hydrie 2010, Lin Y & Sun Z 2010).

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as a cellular energy sensor, and plays a crucial role in fatty acid and glucose homeostasis. In skeletal muscle, AMPK stimulates mitochondrial fatty acid oxidation and ATP production (Cantó *et al*. 2013). AMPK is activated via phosphorylation as a result of metabolic responses, which leads to a direct increase in glucose uptake in skeletal muscle by inducing the translocation of glucose transporter 4 (GLUT4) to the plasma membrane. However, the molecular mechanism by which AMPK regulates glucose uptake still remains elusive (Cantó *et al*. 2013, Yamauchi *et al.* 2008).

Thyroid Hormones and Insulin Resistance

Through the transcriptional regulation of specific genes, THs have critical roles in the maintenance of glucose homeostasis (Brenta. 2010, sVillicev *et al.* 2007, Chidakel *et al.* 2005). However, TH may also induce non-genomic effects that contribute to cellular metabolism (Weitzel *et al.* 2001, Davis *et al.* 2003). While TH drive metabolism, there remains conflicting evidence in the literature on the mechanisms involved in the regulation of glucose homeostasis (Medina *et al.* 2011, Aguer & Harper 2012). Both hyperthyroidism and hypothyroidism have been associated with complications in insulin signaling and glucose intolerance, an incongruence that is likely associated with differential effects of TH on various tissues (Brenta. 2010, Teixeira *et al.* 2012).

Nonetheless the literature suggests that synergies among T3, glucose, and lipid metabolism exists (Lin & Sun 2011, Kim *et al.* 2002). Exogenous T3 induces insulin-stimulated glucose transport and glycolysis in the muscle (Moreno *et al.* 2011). Furthermore, T3 potentiates insulin signaling, insulin sensitivity, and an increase in insulin synthesis (Lin & Sun 2011). However, links among thyroid hormones, hyperglycemia, and insulin resistance remain elusive.

In peripheral tissues, the genomic effects of TH occur after the intracellular transport of T4, and its deiodination to T3, by either DI1 or DI2. The activity of these enzymes predominately found in skeletal muscle regulates the availability of T3, and in turn, may indirectly regulate insulin signaling and glucose homeostasis. The binding of T3 to its nuclear thyroid hormone receptor, THrβ-1, induces an energetically expensive and relatively time-consuming, transcriptional signaling cascade (Bernal & Refetoff 1977,Weitzel *et al.* 2001, Müller *et al.* 2014, Chidakel *et al.* 2005). One of the genes activated in this process, uncoupling protein 2 (UCP2), may contribute to mitochondrial ATP production and to glucose homeostasis (Toda & Diano 2014). THs can also promote metabolic changes through non-genomic effects, which can be manifested within minutes (as opposed to the longer genomic effects) (Weitzel *et al.* 2001). These non-genomic actions are independent of nuclear uptake of TH and may involve plasma membrane, mitochondrial, or cytoplasm receptors that mediate transcriptional actions (Davis *et al.* 2003).

Additionally, THs may also regulate sirtuin 1 (SIRT1), a NAD+- dependent deacetylase that is involved in glucose homeostasis and insulin secretion. Recent studies have demonstrated that increasing expression of SIRT1 improves insulin secretion and sensitivity, especially in insulin resistance conditions (Moynihan *et al.* 2005, Sun *et al* 2007). However, the direct effects of T4 on muscle SIRT1 during insulin resistance are not well defined. Furthermore, down regulation or knockdown of SIRT1 induces insulin resistance in cells and tissues (Sun *et al.* 2007). SIRT1 may also interact with the TH receptor, THrβ1, which suggests a non-coincidental relationship between SIRT1 and THs (Thakran *et al.* 2013).

Models of Type 2 Diabetes

T2DM has long been studied in obese and non-obese animal models with varying degrees of insulin resistance and/or beta cell failure (King 2012). Since T2DM is commonly paralleled with obesity, a majority of the models are of obese nature. However, obesity in these models can be achieved through induced or natural spontaneous genetic mutations, or by high fat feeding (King 2012). Models are categorized as either monogenic (a single genetic mutation) or polygenic (multiple genetic factors) (King 2012). A few monogenic models used present with mutations in leptin signaling, which is responsible for satiety, and include the Lep $^{ob/ob}$ mouse and the Zucker Diabetic Fatty rat (King 2012, Leiter 2009). Although these models are useful in studying several aspect of diabetes they do not completely replicate the complex array of T2DM complications present in humans. Furthermore, obesity in humans does not develop by a single genetic mutation.

Polygenic models of obesity may better replicate the development of human T2DM; however, there is no wild-type controls and the pathogenesis is more evident in males than females (Leiter 2009). Some include the KK mice, which is mildly obese and develops severe hyperinsulinaemia and depicts insulin resistance in muscle and adipose tissue (King 2012). Another polygenic model is the Otsuka Long Evans Tokushima Fatty (OLETF) rat, which was developed from an out-bread colony of Long Evans Tokushima Otsuka (LETO) rat and displays a mutation in the cholecystokinin (CCK) receptor resulting in impaired satiety signaling and diet-induced obesity (DIO) (King 2012, Moran & Bi 2006). The OLETF rat has been extensively studied as a model of human, non-insulin dependent diabetes mellitus, and is characterized by DIO, late onset hyperglycaemia, hyperlipidemia, insulin resistance, and hypertension (Moran and Bi 2006, Montez *et al.*, 2012, Rodriguez *et al.* 2012). This model develops insulin resistance and metabolic syndrome similarly to humans, and the phenotype is displayed in both male and female rats. The OLETF strain also has a healthy LETO counterpart making it an appropriate model to elucidate the pathogenesis of T2DM (Moran & BI, 2006, Rodriguez *et al*, 2012). Despite being an excellent model for T2DM, no studies have been conducted to address the contribution of TH to impaired metabolism in OLETF.

Research Aims

Incongruences in the literature on the relationship between TH and glucose homeostasis during insulin resistance conditions exist. Furthermore, the simultaneous contributions of exogenous T4 on UCP2 and SIRT1 to improved glucose tolerance are not well defined. To address the effects of TH on glucose intolerance we chronically infused insulin resistant, OLETF rats with exogenous T4. Also, because T4 must be deiodinated to T3 before it can mediate a genomic effect, infusion of T4 allows for evaluation of the contribution of deiodinases to impaired metabolism that would otherwise be difficult to assess infusing T3.

Methodology

Animals

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of both Kagawa Medical University (Kagawa, Japan) and the University of California Merced (Merced, CA).

Male lean (265 ± 7 g), strain-control Long Evans Tokushima Otsuka (LETO) rats and obese (356 ± 4 g) Otsuka Long Evans Tokushima Fatty (OLETF) rats (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan), both 9 weeks of age, were assigned to the following groups (n=8/group): 1) untreated LETO, 2) untreated OLETF, and 3) OLETF + T4 (8.0 µg/100g BM/d x 5 wk). Rats were housed in groups of two or three in a specific pathogen-free facility (at Kagawa Medical University, Japan) under controlled temperature (23°C) and humidity (55%) with a 12 hr light and dark cycle. Animals were provided water and food *ad libitum*.

Osmotic minipumps (Alzet, model 2006, Durect Corp., Cupertino, CA) loaded with T4 (Sigma-Aldrich, St. Louis, MO) dissolved in 6.5 mM NaOH and 50% propylene glycol, were implanted subcutaneously, delivering a predefined dose (Klieverik *et al.* 2009).

Body Mass and Food Intake

Body mass (BM) and food intake were measured daily. Sum of cumulative changes in body mass ($\Sigma\Delta_{BM}$) was determined as the sum of consecutive changes in mean daily BM. Changes in $\Sigma\Delta_{BM}$ were determined by comparing slopes of the liner regressions for each group.

Blood Pressure

Systolic blood pressure (SBP) was measured weekly in conscious rats by tail-cuff plethysmorgraphy (BP-98A; Softron Co., Tokyo, Japan) (Rodriguez *et al.* 2012, Vázquez-Medina *et al.* 2013).

Rectal Temperature

Rectal temperature was measured weekly until the end of the study using a small animal warmer and thermometer (BWT-100A; Bio Research Center, Nagoya, Japan).

Oral Glucose Tolerance Test

A week before the end of the study, all animals were fasted overnight (ca. 12 hr) and oGTTs performed as previously described (Rodriguez *et al.* 2012). The glucose area under the curve ($AUC_{glucose}$) and insulin area under the curve ($AUC_{insulin}$) were calculated by the trapezoidal method and used to calculate the insulin resistant index (IRI) as previously described (Habibi *et al.* 2008).

Dissections and Sample Collection

At the end of the treatment, all animals were fasted overnight (ca. 12 hr) and tissues harvested the subsequent morning. Trunk blood was immediately collected in chilled vials containing 50mM EDTA and protease inhibitor cocktail. Samples were centrifuged (3000x g, 15 min, 4°C), plasma collected in cryovials, and snap frozen in liquid nitrogen. The heart, soleus muscle, and epididymal (epi) and retroperitoneal (retro) fat were rapidly dissected, weighed, and frozen in liquid nitrogen. All samples were stored at -80°C until further analysis.

Plasma Analysis

Plasma insulin was measured using a commercially available kit (Rat Insulin ELISA kit; Shibayagi, Gunma, Japan) (Rodriguez *et al.* 2012). Plasma concentrations of total T4, total T3, and serum concentrations of free T4 were determined using commercial radioimmunoassay (RIA) kits (Coat-A-Count kit; Siemens Healthcare Diagnostics, Los Angeles, CA, USA). Plasma thyroid stimulating hormone (TSH) was determined with a commercially available rat ELISA kit (ALPCO Diagnostics, Salem, NH). Plasma glucose, triglycerides (TG), and nonesterified fatty acids (NEFA) were measured as previously described (Rodriguez *et al.* 2012). All samples were analyzed in duplicate with percent coefficients of variability of <10%.

Quantitative PCR

The mRNA expressions of muscle DI1, DI2, GLUT4, MCT10, SIRT1, THrβ1, and UCP2 were quantified by real-time PCR as previously described (Martinez *et al.* 2016, Vázquez-Medina *et al.* 2013). Values were normalized for the expression of β-actin. Samples were run, with positive and negative controls, on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) in a 20µL reaction containing: 10µl of SYBR Green PCR Master Mix (Applied Biosystems), 6µL water, 0.5µL of each primer (20µmol L^{-1}), and 3µL of cDNA (150ng of RNA sample) (Martinez *et al.* 2014, Vázquez-Medina *et al.* 2013). Relative quantity of mRNA levels was plotted as fold-increase compared with the control group levels using the $2^{-\Delta\Delta C_T}$ method. Primer sequences used are provided in Table 3.

Western Blot

Muscle protein content was assessed by Western blot as previously described (Martinez *et al.* 2016, Rodriguez *et al.* 2012, Vázquez-Medina *et al.* 2013). Membranes were incubated with primary antibodies against AMPKα (1:500, Cell Signaling Technology, Danvers, MA), phospho-AMPKα (Thr172) (1:350, Cell Signaling Technology), insulin receptor-β (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-IGF-I receptor-β (1:500, Cell Signaling Technology), and Na+/K+-ATPase-α (1:1000, Santa Cruz Biotechnology). Membranes were washed and incubated with secondary antibodies IR Dye 680RD donkey anti-mouse, IR Dye 680 RD donkey anti- rabbit, or IR Dye 800CW donkey anti-rabbit (1:20000). Proteins were then visualized using the LI-COR Odyssey Infrared Imaging System and quantified with ImageJ (NIH, Bethesda, MD). In addition

consistency in loading equivalent amounts of total protein was confirmed and normalized by correcting for the densitometry values of Ponceau S staining (Gilda & Gomes 2013).

Statistics

Means (±SE) were compared by ANOVA followed by Fisher's protected least-significant difference post-hoc test and considered significant at $P<0.05$. Glucose tolerance was determined by comparing mean AUC values obtained from the oGTT. All statistical analyses were performed with the SYSTAT 11.0 software (SPSS, Richmond, CA).

Results

T4 did not alter food consumption or body composition

Daily food consumption and body mass, and end-of-study tissue masses were measured to assess the potential effects of infused T4 on body composition and food intake. Mean food intake was higher in OLETF than in LETO (27.6 ± 0.3 vs. 19.4 ± 0.3 g/d), but there was no significant treatment effect (27.4±0.4 g/d) (Figure 1A). BM increased by 25% in untreated OLETF compared to LETO, but T4 treatment had no significant effect (Figure 1B). While the slope for the $\Sigma\Delta_{BM}$ regression for LETO (3.0±0.13) was lesser than that for OLETF groups, no significant treatment-effect was observed (4.4 ± 0.19 vs. 4.1 ± 0.16) (Figure 1C). While relative heart and soleus masses were not different among the groups, the fat masses (epi and retro) were greater in the OLETF groups than LETO; however, T4 treatment had no additional effect (Table 1).

T4 did not alter SBP or core-body temperature

SBP was measured to determine if improvements in metabolic defects translated to improvements in arterial blood pressure. Mean SBP in obese OLETF increased by 15% compared to lean LETO (138 ± 2 vs. 117 ± 1 mm HG), and treatment with T4 had no additional effect (140 ± 2 mmHG). These results demonstrate the development of elevated arterial blood pressure in the insulin resistant phenotype, and the lack of improvement with the improved metabolic phenotype associated with the exogenous T4 infusion. Rectal temperatures were also measured to determine the potential thermogenic effects of infused T4. There were no consistent strain or treatment effects on core-body temperature suggesting that the T4-mediated cellular effects had none to minimal thermogenic impacts (Figure 7).

T4 improved glucose intolerance and IRI

An oGTT was conducted to determine the benefits of T4 treatment on glucose intolerance and IRI during an insulin resistant condition. Mean $AUC_{glucose}$ increased by 55% in OLETF compared to LETO, and decreased 15 % in T4-treated OLETF (Figure 2A). Mean $AUC_{insulin}$ increased by 68% in OLETF compared to LETO, and decreased 24% in T4-treated OLETF (Figure 2B). The IRI increased 86% in OLETF compared to LETO, and decreased 34% in T4-treated OLETF (Figure 2C). These results suggest that T4 improved glucose intolerance, insulin clearance, and IRI.

The obese, insulin resistant phenotype is associated with hyperthyroidism

TSH, total T4, free T4, and total T3 were measured to determine the thyroid hormone profile of the insulin resistant animals. TSH was 15% greater in OLETF compared to LETO, and was reduced 8% with T4 treatment (Figure 3A). The other thyroid hormones were consistently greater in OLETF compared to LETO (Figure 3B, 3C, 3D). In addition, tT4 levels were exacerbated with the infusion of T4 as expected and confirming the successful infusion of exogenous T4. Furthermore, the additional increase in fT4 levels

likely reflect an increase in DI2 activity. To further demonstrate the cellular effectiveness of the T4 infusion, Na^{+}-K^{+} ATPase protein expression, a sensitive marker of T4 activity, was measured. The protein expression of Na^{+}-K^{+} ATPase increased nearly 3-fold in the treated OLETF group compared to its untreated counterpart (Figure 4). A 50% reduction in protein expression was associated with the obese OLETF compared to the lean LETO (Figure 4).

T4 increases muscle mRNA expressions of MCT10, DI2, UCP2, and SIRT1
To assess the effects of T4 on the mRNA expressions of key regulators of TH-mediated signaling and metabolism in muscle, MCT10, DI1/2, THrβ1, UCP2, and SIRT1 were measured. Muscle MCT10 mRNA expression decreased over 50% in untreated OLETF compared to LETO, and levels were completely recovered with T4 (Figure 5A). While the decreases in DI2 with OLETF did not reach significance, DI1 levels were significantly reduced (Figure 5B, 5C) and DI2 levels nearly doubled with T4 (Figure 5C). No strain- or treatment-effect was detected in THrβ1 expression (Figure 5D). While no strain-effect in UCP2 and SIRT1 expression levels was detected, T4 increased levels 40% (Figure 5E) and 2.7-fold (Figure 5F), respectively.

T4 improvements in IRI are independent in static changes in insulin signaling
The phosphorylation of AMPK and insulin receptor were measured to help determine the contributing factors to the improvements in glucose intolerance and IRI with T4 infusion. No profound changes in phosphorylation of insulin receptor or AMPK were detected (Figure 6A and 6B). While mean AMPK phosphorylation decreased 30% with T4 treatment, it did not reach significance (Figure 6B). GLUT4 mRNA expression did not exhibit a strain-effect, but T4 reduced levels by 31% (Figure 6C). Collectively this data suggest that the improvements in glucose clearance and IRI are independent of static enhancements in insulin signaling.

T4 does not improve the strain-associated effect on lipid metabolism
Fasting plasma TG and NEFA were measured to assess the effects of T4 treatment on lipid metabolism. Fasting plasma TG (85±9 vs. 36±5 mg/dl) and NEFA (0.78±0.09 vs. 0.45±0.04 mEq/l) increased in OLETF compared with LETO. However, circulating lipids (79.1±11.0mg/dl and 0.67± 0.01mEq/l) were not significantly altered with T4 (Table 2)

Discussion

Hyper- and hypothyroidism are both associated with insulin resistance suggesting that thyroid hormones may have little if any effect on the regulation of glucose during insulin resistance. In the present study we investigated the role exogenous T4 has on glucose intolerance and insulin signaling in a model of insulin resistance. We demonstrated that the infusion of T4 significantly improved the insulin resistant condition and glucose intolerance in the insulin resistant OLETF rat.

Na^+-K^+ ATPase increases with T4 infusion

Na^+-K^+ ATPase is a downstream target of T4 that is responsible for an electrochemical gradient that provides energy for the transport of many ions, metabolites, and nutrients such as glucose (Forst *et al* 2000). The up-regulation of Na^+-K^+ ATPase also imposes an energetic burden on cellular metabolism. Typically diabetes-induced metabolic changes are also associated with alteration to the Na^+-K^+ ATPase activity. For example, impaired skeletal muscle glucose uptake in high fat diet, insulin resistant Wistar rats was associated with a reduction in Na^+-K^+ ATPase, and increased T3 increased its activity (Ho 2011, Lei *et al.* 2004). In the present study the muscle content of Na^+-K^+ ATPase was statically lower in the OLETF rat, and exogenous T4 more than completely recovered the suppressed protein content suggesting that the T4 infusion was effective at inducing a cellular response. Furthermore, the increase in muscle Na^+-K^+ ATPase likely contributed to the improvement in IRI in the T4-treated group by increasing cellular metabolism independent of insulin and AMPK signaling. Additionally, increased Na^+-K^+ ATPase activity is associated with increased SIRT1 (Yuan *et al.* 2014), similar to the relationship demonstrated in the present study. Thus, increasing cellular metabolism independent of enhanced insulin signaling may produce greater benefits on ameliorating obesity-associated insulin resistance than directly targeting insulin-mediated mechanisms.

MCT10 and MCT8 Increase influx of TH

The transport of T4 into the cell is required regardless if the cellular outcomes are genomic or non-genomic. While originally the transport of TH was thought to be passive, multiple membrane-bound transporters have been recently identified including MCT8 and MCT10 suggesting that TH-mediated effects may be regulated by the presence or up-regulation of these specific transporters. Thus, the concentration of thyroid hormone within the cell will determine the rate of cellular regulation of TH-mediated signaling (Van Der Deure *et al.* 2010, Visser 2010). In the present study, MCT10 was reduced and associated with elevated plasma levels of T4 in insulin resistant OLETF rats suggesting that the elevated plasma levels are attributed to reduced cellular transport, and consequently contribute to impairment of the HPT axis. Furthermore, the reduced MCT10 levels may also explain the decreasing trends in the mRNA expression levels of DI1 and DI2 as reduced availability of T4 for conversion to T3 reduces the demand for deiodinases. This is corroborated by reduced protein content of Na+-K+ ATPase, a surrogate marker of energetic demand and downstream target of TH signaling. Conversely, exogenous T4 reversed these strain-associated defects, and likely contributing to the improvements in glucose intolerance and IRI. The recovery of the

MCT10 expression levels, while not associated with a decrease in plasma T4 due to the constant infusion, was associated with increased DI2 and Na+-K+ ATPase. Increased expression of the transporter should translate to a greater availability of T4 inside the cell, and with increased DI2 expression (and likely activity given the increase in fT4), there would be increased conversion to the more biologically active T3. This is supported by the complete recovery of muscle Na^+-K^+ ATPase.

DI2 mRNA increases T3 availability

With skeletal muscle accounting for about 40-50% of the total body mass, it's the largest contributor to resting energy expenditure and insulin-induced glucose utilization (Marsili *et al.* 2010). Furthermore, DI2 has been shown to have a greater effect on T3-dependent gene transcription than D1 (Maia *et al.* 2005). Subjects with genetic defects to DI2 have reduced glucose turnover and DI2 knockout mice become insulin resistant (Hong *et al.* 2013, Maia *et al.* 2005). The present study would corroborate that further demonstrating that exogenous T4 is effective at restoring this insulin resistance-associated defect in DI1/2. In addition DI2 has been shown to provide a protective effect against diet-induced obesity associated glucose dysregulation (Marsili *et al.* 2011), which would explain the increase in mRNA of DI2 in our treated, diet-induced, obese insulin resistant OLETF rats. The increased mRNA expression of the transporter and DI2 can be linked with a higher availability of the T3 inside the cell in order to potentiate the increases of downstream targets that contributed to the improvement in glucose intolerance and insulin resistance.

Static AMPK and INS independent of glucose intolerance improvement

In muscle, the genomic effects of T3 are mediated by its receptor, THrβ1. Furthermore, T3 can increase the expression of THrβ1, which may activate (phosphorylate) AMPK, a key regulator of cellular metabolism (Cantó & Auwerx 2013, Wang *et al.* 2014). When active, AMPK triggers the translocation of GLUT4 to the plasma membrane to increase the uptake of glucose (Cantó & Auwerx 2013). GLUT4 is an insulin-regulated protein that contributes to whole-body glucose homeostasis, and therefore is a key target for understanding insulin resistance in type 2 diabetes mellitus (T2DM) (Teixeira *et al.* 2012). However, in this present study there was no detectable increase in the static activations of muscle insulin receptor or AMPK, or mRNA expression of GLUT4 suggesting that the improvements in insulin resistance with infused T4 was independent of static insulin-mediated signaling. The lack of an increase in THrβ1 would also suggest that the improvements in glucose tolerance were either mediated through: (1) non-genomic mechanisms such as increased cellular metabolism, and/or (2) genomic effects that did not necessitate static elevations in THrβ1 expression. That is, the increase in T4 transport (via increased MCT10) and deiodination (via increased DI2) in the T4 infused rats was likely sufficient to maintain the levels of THrβ1 expression to facilitate the increase in cellular metabolism that translated to improved glucose tolerance. Furthermore, T3 can rapidly increase glucose uptake independent of increased GLUT4 at the cell surface (Teixeira *et al.* 2012), thus the increase in DI2 likely increased the cellular levels of T3 to facilitate glucose uptake independent of GLUT4.
Sirt1, a downstream target of AMPK, is thought to be nutritionally regulated and low levels have been reported with high fat and hyperglycemic conditions (Brandon *et al.*

2015). AMPK typically regulates SIRT1 expression via NAD+ content; however, studies have shown that phosphorylation of AMPK had no effect on SIRT1 levels when knocked out or overexpressed. Furthermore, T3 stimulation enhanced the transcription of genes involved in mitochondrial fatty acid oxidation and gluconeogensis via up-regulation of SIRT1 (Brandon *et al.* 2015, Thakran *et al.* 2013). This is consistent with our findings that demonstrated a decrease in SIRT1 in OLETF rats that were completely recovered by the infusion of T4. Furthermore, the increase in muscle SIRT1 expression with T4 treatment was likely independent of AMPK activation. Thus, the improvements in insulin resistance in the treated group may be the result of non-TH-mediated genomic effects mediated in part by SIRT1. This is supported by a study demonstrating that SIRT1 can directly bind DNA bound transcription factors, including nuclear receptors, and influence transcription (Suh *et al.* 2013).

UCP2 increases associated with IRI improvements

UCP2 is also a downstream target of THrβ1. Although the mechanisms describing UCP2 regulation of glucose metabolism is not well understood, an important link between the two is apparent. For example, subjects with a polymorphism in the transcription of UCP2 are associated with obesity and T2DM (Toda & Diano 2014). In addition, UCP2 increases glucose-stimulated insulin secretion in pancreatic beta cells (Toda & Diano 2014). The insulin resistance phenotype in OLETF is associated with an approximately 20% decrease in hepatic UCP2 protein content, which is more than completely restored with ARB treatment and associated with improved IRI (Montez *et al.* 2012). Similarly, the present study demonstrates a substantial increase in muscle UCP2 expression with infused T4 and an associated improvement in IRI suggesting that the maintenance and/or restoration of systemic UCP2 is an important factor in abating the manifestation and consequences of insulin resistance. Additionally, as with the current study, the previous improvements in IRI were independent of static improvements in the insulin signaling cascade suggesting that maintenance and/or restoration of UCP2 contributes to improvement in IRI independent of direct insulin-mediated signaling (Rodriguez *et al.* 2012).

No deleterious effects of T4 Infusion

A major concern of clinical hyperthyroidism is the deleterious effects associated with potential thyrotoxicosis (Araujo *et al.* 2008, Brenta. 2010, Villicev *et al.* 2007). Exogenous T4 treatment did not have any deleterious effects on the body composition, food consumption, excessive thermogenesis, and SBP suggesting that thyrotoxicosis was not a compounding factor. As expected, exogenous T4 administration decreased TSH levels and increased plasma T4, indicative of intact negative feedback mechanism and successful accomplishment of chronic elevation of circulating T4 with the infusion. The untreated OLETF was also characterized by relative hyperthyroidism with elevated triglycerides and non-esterified fatty acids suggesting that thyroid hormones do not profoundly improve the dyslipidemia associated with insulin resistance. Furthermore, the OLETF model presents with an impaired hypothalamic-pituitary-thyroid negative feedback mechanism as the nearly doubled T4 levels are associated with a 15% increase in circulating TSH. This impaired negative feedback is restored in the T4 infused rats.

Additionally, hyperthyroid states may be associated with elevated plasma free fatty acids and glucose levels, which possibly contribute to the decrease in insulin sensitivity during this condition. However, it remains unclear if this condition is a cause or a consequence of insulin resistance in hyperthyroidism (Teixeira *et al.* 2012).

Summary

In conclusion, we found that exogenous T4 treatment improved glucose intolerance and insulin resistance via increased cellular metabolism. The increase in cellular metabolism was achieved by increases in the mRNA expressions of MCT10, DI2, SIRT1, and UCP2 that likely resulted in increased content of muscle Na^{+}-K^{+} ATPase. The results suggest that T4 treatment increased the influx of T4 in skeletal muscle and, with an increase of DI2, increased the availability of the biologically active T3 to up regulate key factors such SIRT1 and UCP2 involved in cellular metabolism and glucose homeostasis. Furthermore, these changes in cellular events are largely suppressed in the obese, insulin resistant OLETF rat suggesting that these factors contribute to the manifestation of metabolic syndrome in this model. The ability of exogenous T4 to ameliorate this condition is independent of the phosphorylation (activation) of insulin receptor and AMPK, which combined translated into unaltered GLUT4. These results collectively suggest that impaired thyroid hormone regulation of key factors in cellular metabolism contributes to the glucoses intolerance associated with insulin resistance and ultimately the development of metabolic syndrome.

Perspectives

Typically defects in insulin signaling are the primary characteristics to T2DM, and thus, the main focus of understanding of its pathogenesis. However, in this present study we provide an alternate approach to improving glucose intolerance and insulin resistance by targeting TH-mediated metabolism as opposed to insulin targeting. Here we show that through an increase in the availability of the biologically active T3 we are able to stimulate cellular metabolism through key factors in glucose homeostasis. However, the measurements in this study were only conducted in peripheral muscle tissues, parallel measures in other tissues such as liver and adipose, which are also responsible for glucose mechanisms.

Future Directions

In the present study we observed an improvement in glucose intolerance and insulin resistance via a mechanism independent of insulin signaling. This improvement is not associated with an increase in GLUT4 that suggests glucose must be transported into the cell via other mechanisms. Families of Na+-dependent glucose co-transporters, SGLT (sodium/glucose cotrasnporter) have been associated with providing an alternate mechanism for glucose transport. The Na^{+}-K^{+} ATPase pump generates an electrochemical gradient to facilitate the transport of glucose into cells against a concentration gradient (Wood & Trayhurn 2003). Specifically hSGLT3 is expressed in skeletal muscle and provides a secondary active transport of glucose across the muscle

membrane and is insulin-independent (Castaneda *et al.* 2006). In this study we show an increase in the Na^+-K^+ ATPase protein expression, which leads us to believe this is associated with an increase in glucose influx into the cell via the Na+- dependent hSGLT3. Therefore, measuring this co-transporter would further elucidate the mechanism of glucose homeostasis seen with a T4 treatment.

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Tables

Table 1. Relative masses (g/100g BM) of heart, soleus muscle, and epi and retro fat in LETO, OLETF, and OLETF+T4 after 5 wk treatment (n=8/group).

	LETO	OLETF	OLETF+T4
Relative heart mass	0.38±0.03	0.29±0.01	0.29±0.01
Relative soleus muscle mass	0.05±0.008	0.03±0.001	0.03±0.002
Relative epi fat mass	0.94±0.06	1.4±0.09†	1.4±0.11†
Relative retro fat mass	1.3±0.07	3.1±0.17†	2.8±0.18†

† $P<0.05$ vs. LETO

Table 2. Mean (±SE) fasting plasma biochemical measurements after 5 wk of treatment in LETO, OLETF, and OLETF+T4; n=8. *P<0.05 vs. LETO

	LETO	OLETF	OLEF+T4
TG(mg/dl)	35.7±5.3	84.5±8.7†	79.1±11.0
NEFA(mEq/liter)	0.45±0.04	0.78±0.09†	0.67±0.08

† P<0.05 vs. LETO

Table 3. Primer sequences used for real-time PCR

PRIMER NAME	Sequence (5'-3')
GLUT4 FW	CCCACCAGACTCCCTCCTTTCC
GLUT4 RV	GCCACAGCCTAGCCACAACAC
DIO1 FW	TTGACCAGTTCAAGAGACTCGTAGA
DIO1 RV	GTTCTTAAAAGCCCATCCATCTGT
DIO2 FW	CTTTGAACGTGTGTGCATCGT
DIO2 RV	TCTCCAGCCAACTTCGGACTT
UCP2 FW	AAGACCATTGCACGAGAGGAA
UCP2 RV	TAGGTCACCAGCTCAGTACAGTTGA
SIRT1 FW	TTTCAGAACCACCAAAGCG
SIRT1 RV	TCCCACAGGAAACAGAAACC
MCT10 FW	TGTTCGGCTGCCGGAGAACA
MCT10 RV	TGACCAGTGACGGCTGGTAG
THRB1 FW	AGCCAGCCACAGCACAGTGA
THRB1 RV	CGCCAGAAGACTGAAGCTTGC
Beta-actin FW	GTGCTATGTTGCCCTAGACTTCG
Beta-actin RV	GATGCCACAGGATTCCATACCC

Figures

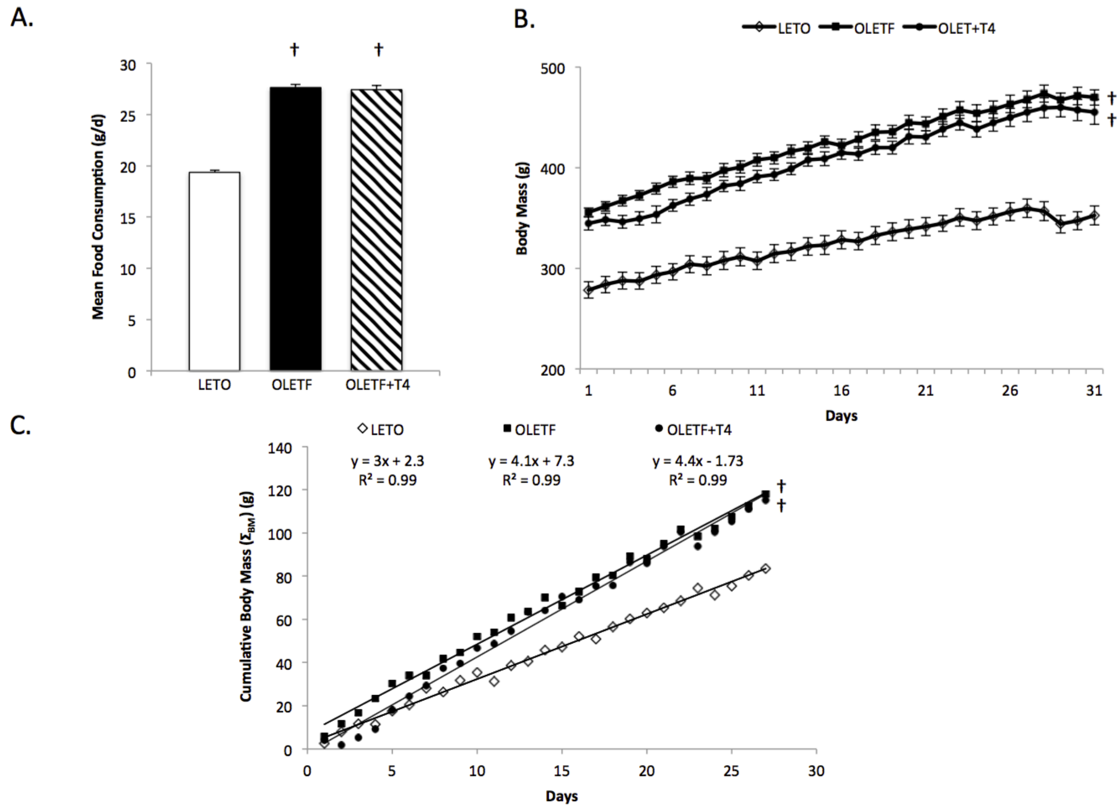

Figure 1. Mean (±SE)(A) food consumption, (B) BM throughout the course of the study, and (C) cumulative change of BM of LETO, OLETF, and OLETF+T4. †, P<0.05 vs. LETO.

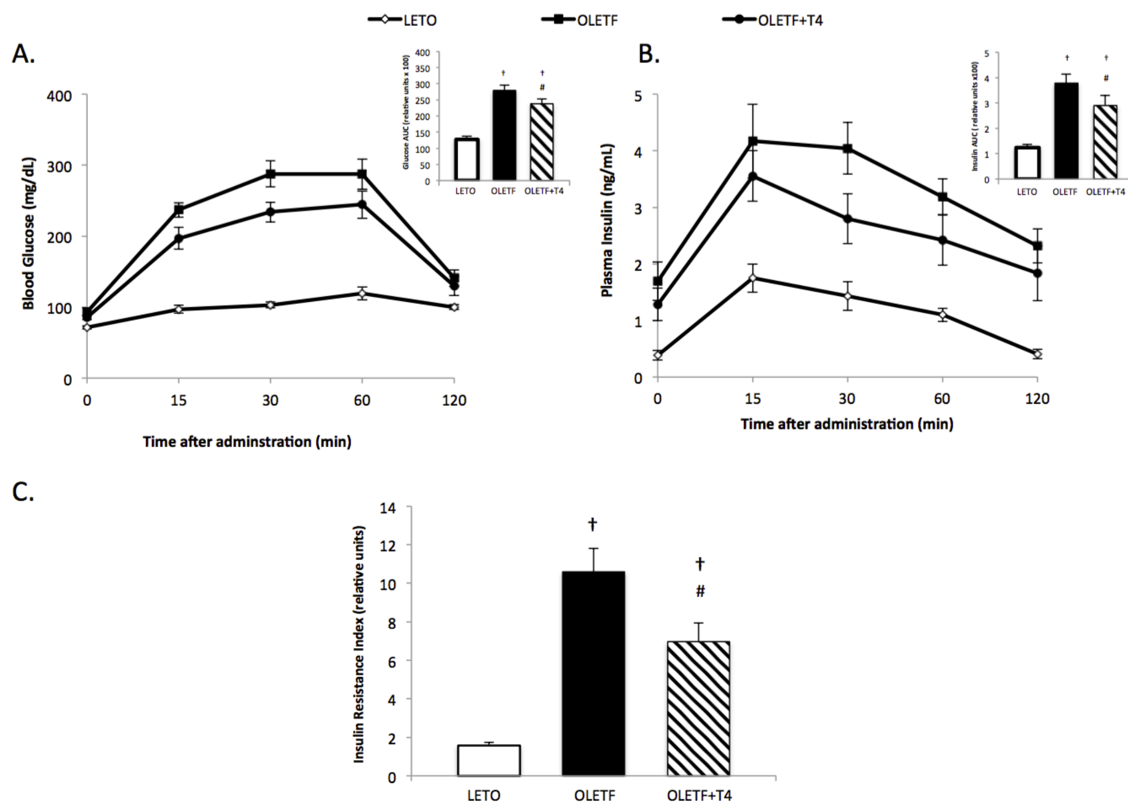

Figure 2. Mean (±SE) (A) blood glucose and (B) plasma insulin levels taken during an oral glucose tolerance test and corresponding area under the curve (AUC) and (C) insulin resistance index (IRI) of LETO, OLETF, and OLETF+T4. †, $P<0.05$ vs. LETO. #, $P<0.05$ vs. OLETF.

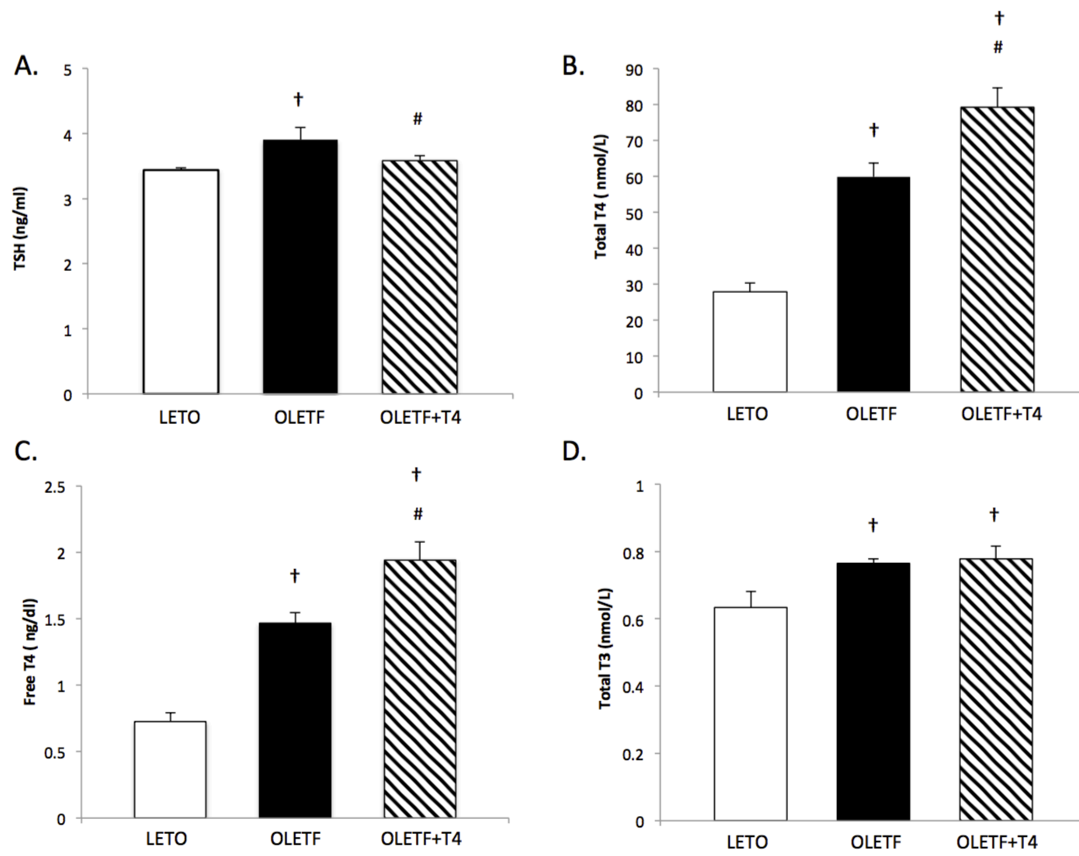

Figure 3. Mean (±SE) (A) TSH, (B) total T4, and (C) free T4 (fT4), and (D) total T3 measured in plasma of LETO, OLETF, and OLETF+T4. †, P<0.05 vs. LETO. #, P<0.05 vs. OLETF.

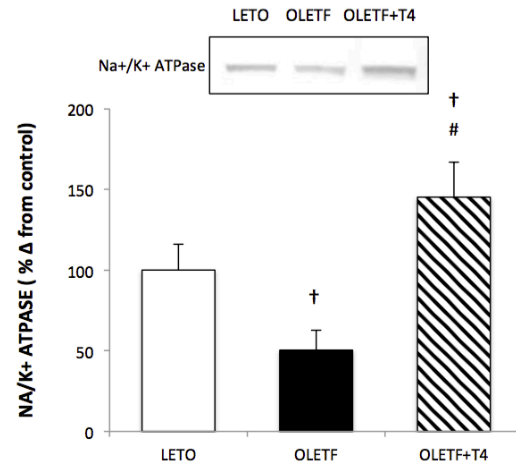

Figure 4. Mean (±SE) percent change from LETO for Na+/ K+ ATPase in soleus muscle of LETO, OLETF, and OLETF+T4 after 5 wk treatment and representative Western blot bands. †, $P<0.05$ vs. LETO. #, $P<0.05$ vs. OLETF.

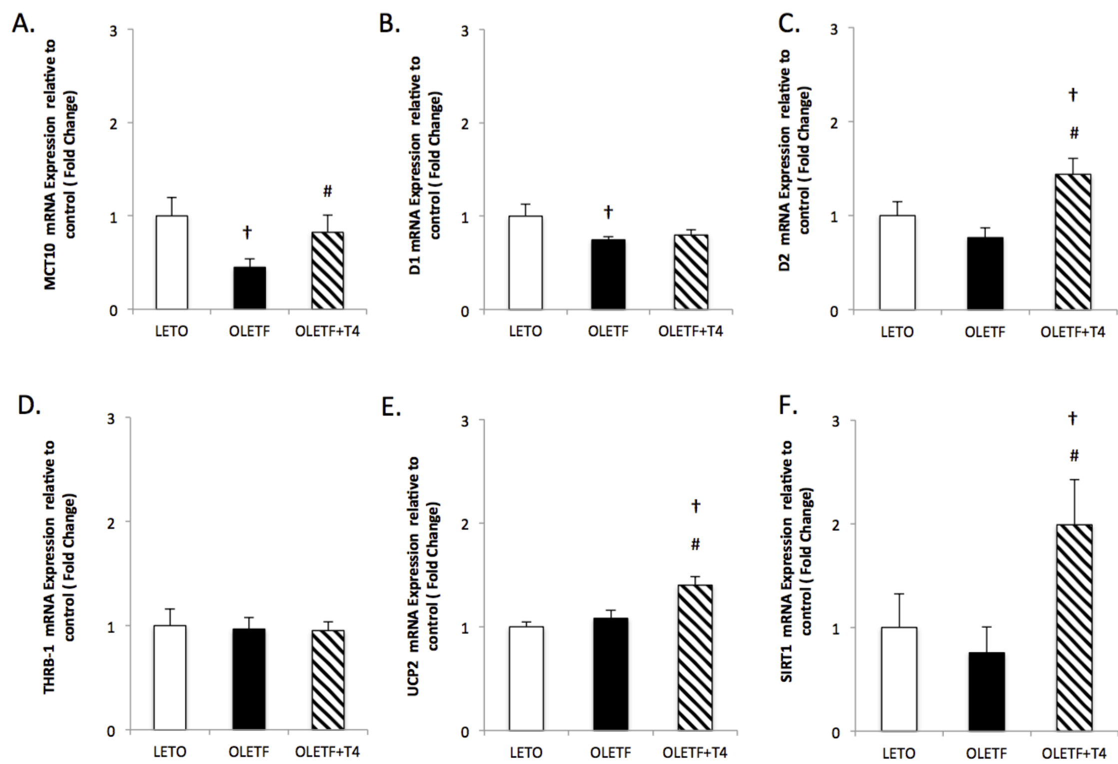

Figure 5. Mean (±SE) fold change of mRNA expression relative to LETO for (A) MCT10, (B) DI1, (C) DI2, (D) THRβ-1, (E) UCP2, and (F) SIRT 1 in soleus muscle of LETO, OELTF, and OLETF+T4 after 5 wk treatment. †, $P<0.05$ vs. LETO. #, $P<0.05$ vs. OLETF.

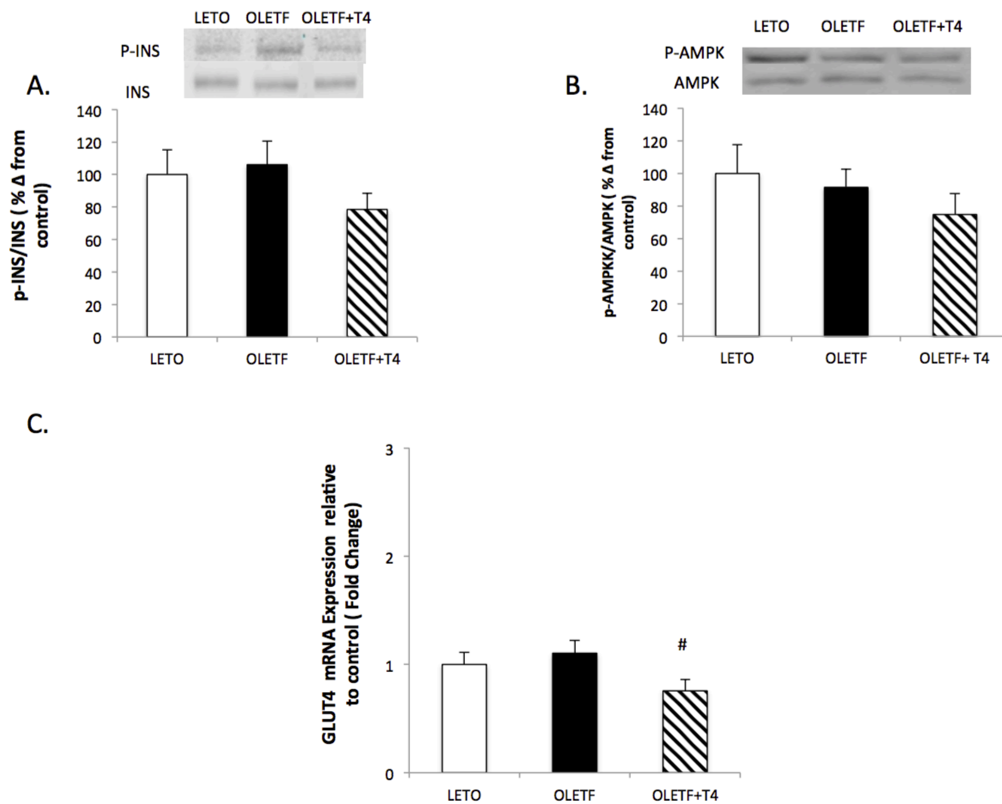

Figure 6. Mean (±SE) percent change from LETO for the ratios of (A) p-INS to INS (B) p-AMPK to AMPK including representative Western blot bands and (C) mean fold change of mRNA expression relative to LETO for GLUT 4 in soleus muscle of LETO, OLETF, and OLETF+T4 after 5 wk treatment. #, P<0.05 vs. OLETF.

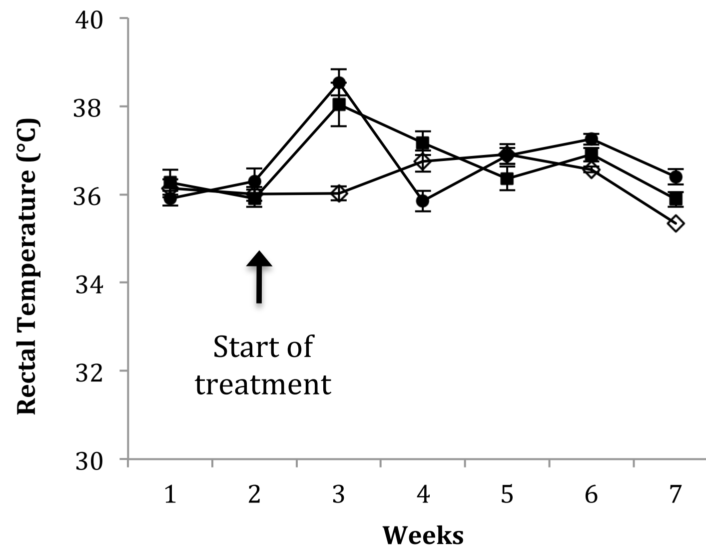

Figure 7. Mean (±SE) weekly rectal temperatures of LETO, OLETF and OLETF+T4. Treatment began on week two.