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Research

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Thyroxine improves glucose intolerance

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Exogenous thyroxine improves glucose intolerance in insulin-resistant rats

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Abstract

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 glucoregulatory effects during conditions of impaired metabolism are not well described. To assess the effects of thyroxine (T_4) 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 + T_4 (8.0 µg/100 g BM/day × 5 weeks). T_4 attenuated glucose intolerance by 15% and decreased IR index (IRI) by 34% in T_4-treated OLETF compared to untreated OLETF despite a 31% decrease in muscle *Glut4* mRNA expression. T_4 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 T_4 treatment increased the influx of T_4 in skeletal muscle and, with an increase of DI2, increased the availability of the biologically active T_3 to upregulate key factors such SIRT1 and UCP2 involved in cellular metabolism and glucose homeostasis.

Key Words

- ► thyroxine
- ► glucose intolerance
- ► insulin-resistant rat
- ► OLETF rat

Introduction

Thyroid hormones (TH) have a multitude of physiological effects related to thermogenesis, metabolism, heart rate and body composition (Araujo *et al.* 2008, Lin & Sun 2011). Through the transcriptional regulation of specific genes, THs have critical roles in the maintenance of glucose homeostasis (Chidakel *et al.* 2005, Villicev *et al.* 2007, Brenta 2010). However, evidence suggests that TH may induce non-genomic effects that contribute to

cellular metabolism (Weitzel *et al.* 2001, Davis & Davis 2003). Although it is clearly established that TH drives 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, a paradox 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 T_3, glucose and lipid metabolism exist (Kim *et al.* 2002, Lin & Sun 2011). Exogenous T_3 induces insulin-stimulated glucose transport and glycolysis in the muscle (Moreno *et al.* 2011). Furthermore, T_3 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 the predominate TH, thyroxine (T_4) and its deiodination to triiodothyronine (T_3), by either deiodinase type 1 (DI1) or type II (DI2). The activity of these enzymes predominately found in skeletal muscle regulates the availability of T_3, and in turn, may indirectly regulate insulin signaling and glucose homeostasis. The binding of T_3 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, Chidakel *et al.* 2005, Müller *et al.* 2014). 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 & Davis 2003). 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 T_4, potentiating non-genomic effects (Van Der Deure *et al.* 2010).

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 T_4 on muscle SIRT1 during insulin resistance are not well defined. Furthermore, downregulation 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).

Incongruences in the literature on the relationship between TH and glucose homeostasis during insulin resistance conditions exist. Furthermore, the simultaneous contributions of exogenous T_4 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, Otsuka Long Evans Tokushima Fatty (OLETF) rats with exogenous T_4 (OLETF + T_4).

Materials and methods

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, USA).

Animals

Male lean ($265 \pm 7\,g$), strain-control Long Evans Tokushima Otsuka (LETO) rats and obese ($356 \pm 4\,g$) 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 + T_4 ($8.0\,\mu g/100\,g\,BM/day \times 5$ weeks). 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-h light and darkness cycle. Animals were provided water and food *ad libitum*.

T_4 administration

Osmotic minipumps (Alzet, model 2006, Durect Corp., Cupertino, CA, USA) loaded with T_4 (Sigma-Aldrich) dissolved in 6.5 mM NaOH and 50% propylene glycol, were implanted subcutaneously, delivering a predefined dose (Klieverik *et al.* 2008, 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 plethysmography (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. 12h) 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

At the end of the treatment, all animals were fasted overnight (ca. 12h) and tissues were harvested the subsequent morning. Trunk blood was immediately collected in chilled vials containing 50mM EDTA and protease inhibitor cocktail. Samples were centrifuged (3000*g*, 15min, 4°C), plasma was 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 T_4, total T_3 and serum concentrations of free T_4 were determined using commercial radioimmunoassay (RIA) kits (Coat-A-Count kit; Siemens Healthcare Diagnostics). Plasma thyroid-stimulating hormone (TSH) was determined with a commercially available rat ELISA kit (ALPCO Diagnostics, Salem, NH, USA). 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%.

Real-time quantitative PCR

The mRNA expressions of muscle *Di1*, *Di2*, *Glut4*, *Mct10*, *Sirt1*, *Thrb1*, and *Ucp2* were quantified by real-time PCR as previously described (Vázquez-Medina *et al.* 2013, Martinez *et al.* 2016). Values were normalized for the expression of β-actin. Samples were run, with positive
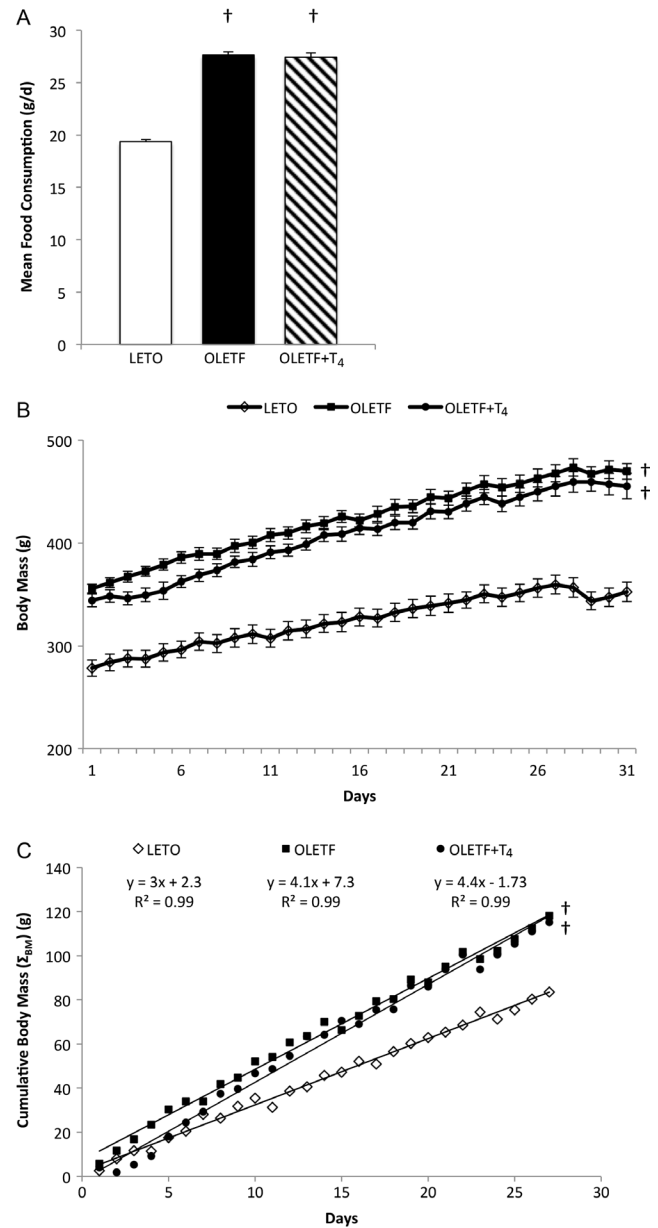

Figure 1
Mean (±s.e.) (A) food consumption, (B) BM throughout the course of the study and (C) cumulative change of BM of LETO, OLETF and OLETF+T_4. †$P < 0.05$ vs LETO.

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and negative controls, on a 7500 Real-Time PCR system (Applied Biosystems) in a 20 µL reaction containing: 10 µL of SYBR Green PCR Master Mix (Applied Biosystems), 6 µL water, 0.5 µL of each primer (20 µmol/L) and 3 µL of cDNA (150 ng of RNA sample) (Vázquez-Medina *et al.* 2013, Martinez *et al.* 2016). 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 Supplementary Table 1 (see section on supplementary data given at the end of this article).

Quantification of protein expression by Western blot

Muscle protein content was assessed by Western blot as previously described (Rodriguez *et al.* 2012, Vázquez-Medina *et al.* 2013, Martinez *et al.* 2016). Membranes were incubated with primary antibodies against AMPKα (1:500, Cell Signaling Technology), phospho-AMPKα (Thr172) (1:350, Cell Signaling Technology), insulin receptor-β (1:500, Santa Cruz Biotechnology), 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 (1:20,000) antibody. Proteins were then visualized using the LI-COR Odyssey Infrared Imaging System and quantified with ImageJ (NIH). 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 (±S.E.) were compared by ANOVA followed by Fisher's protected least-significant difference *post hoc* test and considered significant at $P < 0.05$.

Table 1 Relative masses (g/100 g BM) of heart, soleus muscle and epi and retro fat in LETO, OLETF and OLETF+T$_4$ after 5-week treatment (*n* = 8/group).

	LETO	OLETF	OLETF+T$_4$
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.

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).
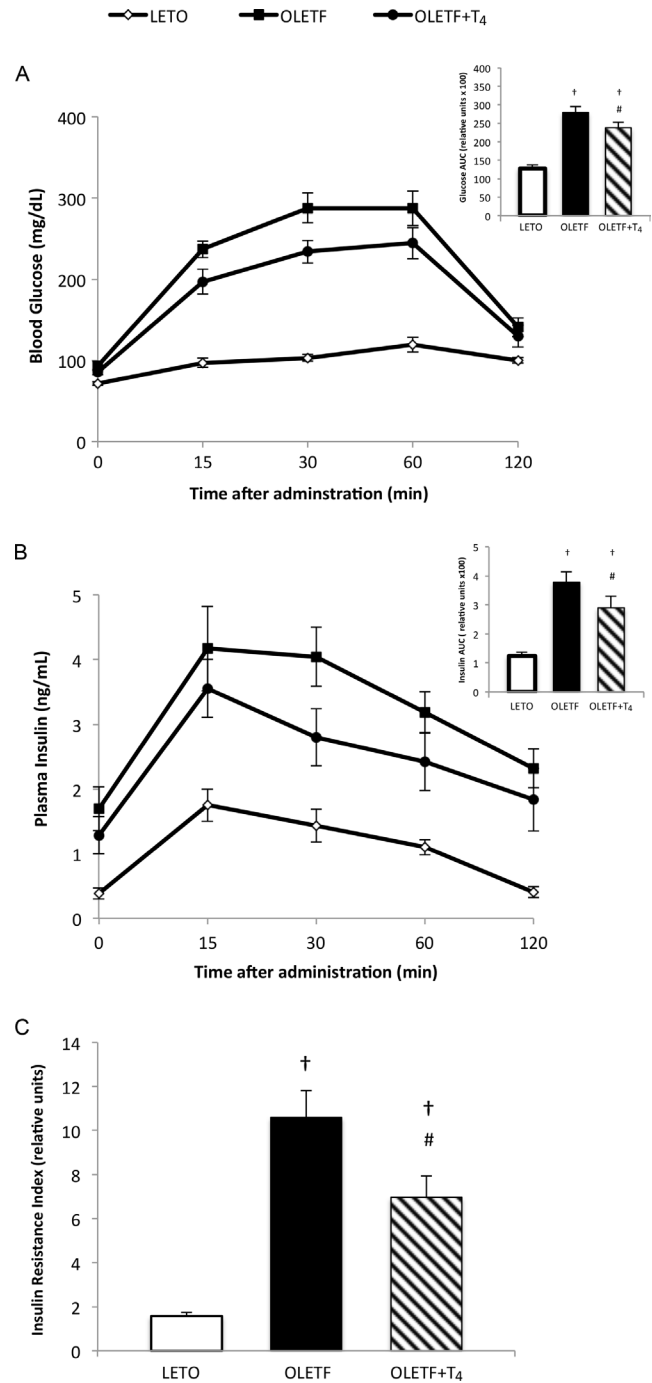
Figure 2
Mean (±S.E.) (A) blood glucose and (B) plasma insulin levels taken during an oral glucose tolerance test and corresponding area under the curve (AUC) and (C) insulin resistance index (IRI) of LETO, OLETF and OLETF+T$_4$. [†]$P < 0.05$ vs LETO. [#]$P < 0.05$ vs OLETF.
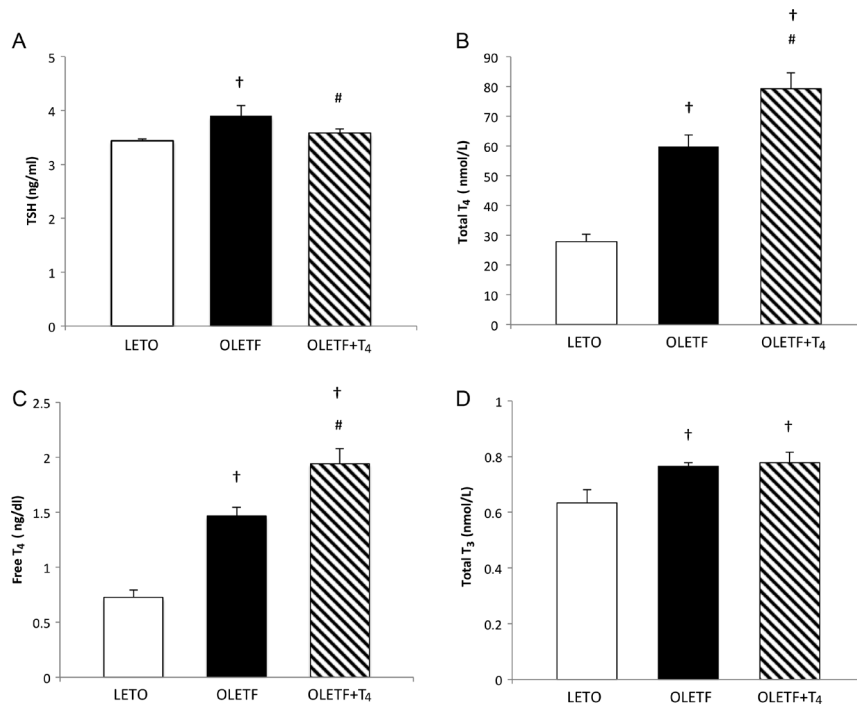
Figure 3
Mean ($\pm$ s.e.) (A) TSH, (B) total T_4, (C) free T_4 (fT4) and (D) total T_3 measured in plasma of LETO, OLETF and OLETF + T_4. $^\dagger P < 0.05$ vs LETO. $^\# P < 0.05$ vs OLETF.

Results

T_4 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 T_4 on body composition and food intake. Mean food intake was higher in OLETF than that in LETO (27.6 ± 0.3 vs 19.4 ± 0.3 g/day), but there was no significant treatment effect (27.4 ± 0.4 g/day) (Fig. 1A). BM increased by 25% in untreated OLETF compared to LETO, but T_4 treatment had no significant effect (Fig. 1B). Although 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) (Fig. 1C). Although relative heart and soleus masses were not different among the groups, the fat masses (epi and retro) were greater in the OLETF groups than those in the LETO; however, T_4 treatment had no additional effect (Table 1).

T_4 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 mmHG), and treatment with T_4 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 T_4 infusion. Rectal temperatures were also measured to determine the potential thermogenic effects of infused T_4. There were no consistent strains or treatment effects on core body temperature suggesting that the T_4-mediated cellular effects had none to minimal thermogenic effects (Supplementary Fig. 1).

T_4 improved glucose intolerance and IRI

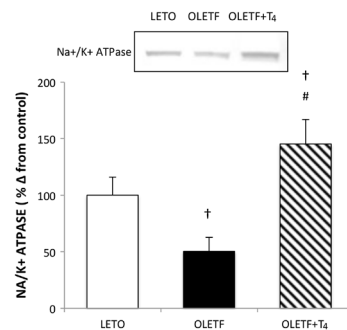An oGTT was conducted to determine the benefits of T_4 treatment on glucose intolerance and IRI during an

Figure 4
Mean ($\pm$ s.e.) percent change from LETO for Na+/K+ ATPase in soleus muscle of LETO, OLETF and OLETF + T_4 after 5-week treatment and representative Western blot bands. $^\dagger P < 0.05$ vs LETO. $^\# P < 0.05$ vs OLETF.

insulin-resistant condition. Mean $AUC_{glucose}$ increased by 55% in OLETF compared to LETO and decreased 15% in T_4-treated OLETF (Fig. 2A). Mean $AUC_{insulin}$ increased by 68% in OLETF compared to LETO and decreased 24% in T_4-treated OLETF (Fig. 2B). The IRI increased 86% in OLETF compared to LETO and decreased 34% in T_4-treated OLETF (Fig. 2C). These results suggest that T_4 improved glucose intolerance, insulin resistance and IRI in the OLETF rat.

The obese, insulin-resistant phenotype is associated with hyperthyroidism

TSH, total T_4, free T_4 and total T_3 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 T_4 treatment (Fig. 3A). Total T_4, free T_4, and total T_3 were consistently greater in OLETF compared to LETO (Fig. 3B, C and D). In addition, fT4 and tT4 levels were exacerbated with the infusion of T_4 as expected, confirming the successful infusion of exogenous T_4. The elevated fT4 levels increase the availability of biologically active hormone to support the increase in DI2 for conversion to intracellular T_3. To further demonstrate the cellular effectiveness of the T_4 infusion, Na^+–K^+ ATPase protein expression, a sensitive marker of T_4 activity, was measured. The protein expression of Na^+–K^+ ATPase increased nearly 3-fold in the treated OLETF group compared to its untreated counterpart (Fig. 4). A 50% reduction in protein expression was associated with the obese OLETF compared to the lean LETO (Fig. 4).

T_4 increases muscle mRNA expressions of *Mct10*, *Di2*, *Ucp2* and *Sirt1*

To assess the effects of T_4 on the mRNA expressions of key regulators of TH-mediated signaling and metabolism in muscle, *Mct10*, *Di1/2*, *Thrb1*, *Ucp2* and *Sirt1* were measured. Muscle *Mct10* mRNA expression decreased over 50% in untreated OLETF compared to LETO, and levels were completely recovered with T_4 (Fig. 5A). Although the decreases in *Di2* with OLETF did not reach significance, *Di1* levels were significantly reduced (Fig. 5B and C) and *Di2* levels nearly doubled with T_4 (Fig. 5C). No strain or treatment effect was detected in *Thrb1* expression (Fig. 5D). Although no strain effect in *Ucp2* and *Sirt1* expression levels was detected, T_4 increased levels 40% (Fig. 5E) and 2.7-fold (Fig. 5F), respectively.

T_4 improvements in IRI are independent in static changes in insulin signaling

The phosphorylation of AMPK and insulin receptor was measured to help determine the contributing factors to the improvements in glucose intolerance and IRI with T_4 infusion. No profound changes in phosphorylation of insulin receptor or AMPK were detected (Fig. 6A and B). Although mean AMPK phosphorylation decreased 30% with T_4 treatment, it did not reach significance (Fig. 6B). *Glut4* mRNA expression did not exhibit a strain effect, but T_4 reduced levels by 31% (Fig. 6C). Collectively, these data suggest that the improvements in glucose clearance and IRI are independent of static enhancements in
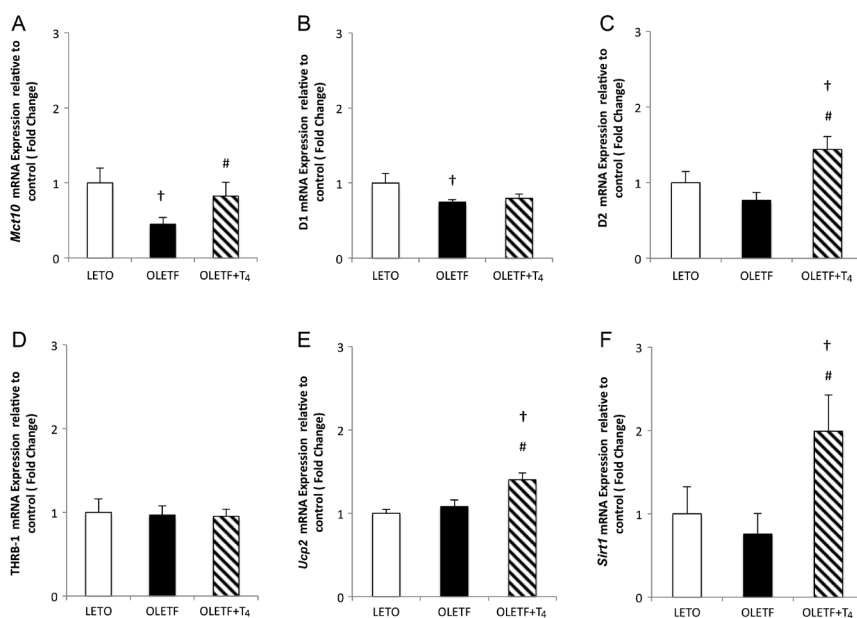

Figure 5
Mean ($\pm$s.e.) fold change of mRNA expression relative to LETO for (A) *Mct10*, (B) DI1, (C) *Di2*, (D) THRβ-1, (E) *Ucp2* and (F) SIRT 1 in soleus muscle of LETO, OELTF and OLETF + T_4 after 5-week treatment. $^{†}P < 0.05$ vs LETO. $^{#}P < 0.05$ vs OLETF.
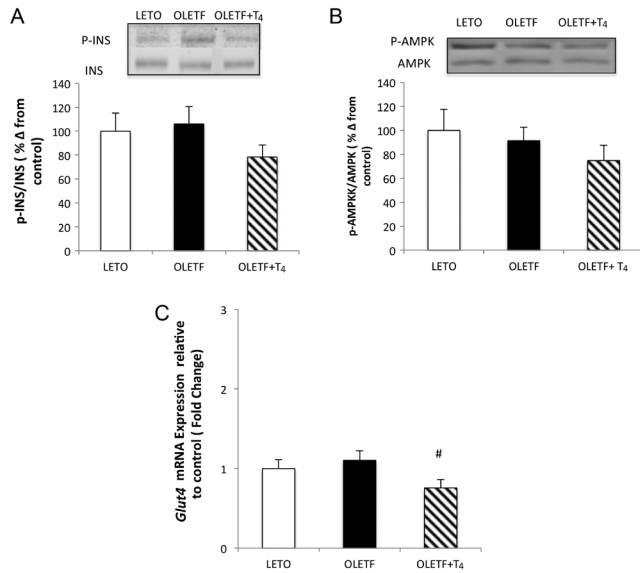
Figure 6

Mean (±S.E.) 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 *Glut4* in soleus muscle of LETO, OLETF and OLETF+T$_4$ after 5-week treatment. $^\#P<0.05$ vs OLETF.

insulin signaling and/or are the result of other, non-traditional signaling pathways that are associated with other glucose transporters.

T$_4$ does not improve the strain-associated effect on lipid metabolism

Fasting plasma TG and NEFA were measured to assess the effects of T$_4$ 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 ± 11.0 mg/dL and 0.67 ± 0.01 mEq/L) were not significantly altered with T$_4$.

Discussion

Hyperthyroidism 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 that exogenous T$_4$ has on glucose intolerance and insulin signaling in a model of insulin resistance. We demonstrated that the infusion of T$_4$ significantly improved the insulin-resistant condition and glucose intolerance in the insulin-resistant OLETF rat.

Na$^+$–K$^+$ ATPase is a downstream target of T$_4$ 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 upregulation 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 T$_3$ increased its activity (Lei *et al.* 2004, Ho 2011). In the present study, the muscle content of Na$^+$–K$^+$ ATPase was statically lower in the OLETF rat, and exogenous T$_4$ more than completely recovered the suppressed protein content suggesting that the T$_4$ 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 T$_4$-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 in ameliorating obesity-associated insulin resistance than directly targeting insulin-mediated mechanisms.

The transport of T$_4$ into the cell is required regardless of whether the cellular outcomes are genomic or non-genomic. Although 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 upregulation 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). In the present study, *Mct10* was reduced and associated with elevated plasma levels of T$_4$ in insulin-resistant OLETF rats suggesting that the elevated plasma levels are attributed to reduced cellular transport, and consequently contribute to the 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 T$_4$ for conversion to T$_3$ 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 T$_4$ reversed these strain-associated defects,

and likely contributed to the improvements in glucose intolerance and IRI. The recovery of the *Mct10* expression levels, although not associated with a decrease in plasma T_4 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 T_4 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 T_3. This is supported by the complete recovery of muscle Na$^+$–K$^+$ ATPase.

With skeletal muscle accounting for about 40–50% of the total body mass, it is 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 T_3-dependent gene transcription than *Di1* (Maia *et al.* 2005). Subjects with genetic defects to *Di2* have reduced glucose turnover and *Di2*-knockout mice become insulin resistant (Maia *et al.* 2005, Hong *et al.* 2013). The present study would corroborate that further demonstrating that exogenous T_4 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 T_3 inside the cell to potentiate the increases of downstream targets that contributed to the improvement in glucose intolerance and insulin resistance.

In muscle, the genomic effects of T_3 are mediated by its receptor, THrβ1. Furthermore, T_3 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 and a reduction of mRNA expression of *Glut4* suggesting that the improvements in insulin resistance with infused T_4 was independent of static enhancements in the traditional insulin-mediated signaling pathway. Alternatively, the improvements

may have been accomplished through the contributions of other glucose transporters not measured here. The lack of an increase in *Thrb1* 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 T_4 transport (via increased MCT10) and deiodination (via increased DI2) in the T_4-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, T_3 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 T_3 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, T_3 stimulation enhanced the transcription of genes involved in mitochondrial fatty acid oxidation and gluconeogensis via upregulation of SIRT1 (Thakran *et al.* 2013, Brandon *et al.* 2015). This is consistent with our findings that demonstrated a decrease in *Sirt1* in OLETF rats that were completely recovered by the infusion of T_4. Furthermore, the increase in muscle SIRT1 expression with T_4 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 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 T_4 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). Furthermore, the observed improvements in glucose metabolism may be attributed to the upregulation of UCP1 in brown adipose tissue (BAT). BAT in rodents has been shown to improve not only the control of thermogenesis but also glucose metabolism (Matsen *et al.* 2013). Furthermore, a human case study demonstrated that T_4 treatment induced brown adipose tissue and ameliorated diabetes in a patient with extreme insulin resistance. This improvement was associated with an increase of UCP1 and DI2 in BAT (Skarulis *et al.* 2010).

A major concern of clinical hyperthyroidism is the deleterious effects associated with potential thyrotoxicosis (Villicev *et al.* 2007, Araujo *et al.* 2008, Brenta 2010). Exogenous T_4 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 T_4 administration decreased TSH levels and increased plasma T_4, indicative of intact negative feedback mechanism and successful accomplishment of chronic elevation of circulating T_4 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 T_4 levels are associated with a 15% increase in circulating TSH. This impaired negative feedback is restored in the T_4-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).

In conclusion, we found that exogenous T_4 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 T_4 treatment increased the influx of T_4 in skeletal muscle and, with an increase of DI2, increased the availability of the biologically active T_3 to upregulate 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 T_4 to ameliorate this condition is independent of the phosphorylation (activation) of the insulin receptor and AMPK, which probably translates to a reduced expression of *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.

References

Aguer C & Harper M-E 2012 Skeletal muscle mitochondrial energetics in obesity and type 2 diabetes mellitus: endocrine aspects. *Best Practice and Research Clinical Endocrinology and Metabolism* **26** 805–819. (doi:10.1016/j.beem.2012.06.001)

Araujo RL, de Andrade BM, de Figueiredo ÁSP, da Silva ML, Marassi MP, Pereira V dos S, Bouskela E & Carvalho DP 2008 Low replacement doses of thyroxine during food restriction restores type 1 deiodinase activity in rats and promotes body protein loss. *Journal of Endocrinology* **198** 119–125. (doi:10.1677/JOE-08-0125)

Bernal J & Refetoff S 1977 The action of thyroid hormone. *Clinical Endocrinology* **6** 227–249. (doi:10.1111/j.1365-2265.1977.tb03319.x)

Brandon AE, Tid-ang J, Wright LE, Stuart E, Suryana E, Bentley N, Turner N, Cooney GJ, Ruderman B & Kraegen EW 2015 Overexpression of SIRT1 in rat skeletal muscle does not alter glucose induced insulin resistance. *PLoS ONE* **10** e0121959. (doi:10.1371/journal.pone.0121959)

Brenta G 2010 Diabetes and thyroid disorders. *British Journal of Diabetes and Vascular Disease* **10** 172–177. (doi:10.1177/1474651410371321)

Cantó C & Auwerx J 2013 AMP-activated protein kinase and its downstream transcriptional pathways. *Cellular and Molecular Life Sciences* **67** 3407–3423. (doi:10.1007/s00018-010-0454-z)

Chidakel A, Mentuccia D & Celi FS 2005 Peripheral metabolism of thyroid hormone and glucose homeostasis. *Thyroid* **15** 899–903. (doi:10.1089/thy.2005.15.899)

Davis PJ & Davis FB 2003 Nongenomic actions of thyroid hormone. In: *Diseases of the Thyroid*, pp 19–37. Totowa, NJ, USA: Humana Press. (doi:10.1007/978-1-59259-352-1_2)

Forst T, De La Tour DD, Kunt T, Pfützner A, Goitom K, Pohlmann T, Schneider S, Johansson BL, Wahren J, Löbig M, *et al*. 2000. Effects of proinsulin C-peptide on nitric oxide, microvascular blood flow and erythrocyte Na+, K+-ATPase activity in diabetes mellitus type I. *Clinical Science* **98** 283–290. (doi:10.1042/cs19990241)

Gilda JE & Gomes AV 2013 Stain-Free total protein staining is a superior loading control to b-actin for Western blots. *Analytical Biochemistry* **440** 186–188. (doi:10.1016/j.ab.2013.05.027)

Habibi J, Whaley-Connell A, Hayden MR, DeMarco VG, Schneider R, Sowers SD, Karuparthi P, Ferrario CM & Sowers JR 2008 Renin inhibition attenuates insulin resistance, oxidative stress, and pancreatic remodeling in the transgenic Ren2 rat. *Endocrinology* **149** 5643–5653. (doi:10.1210/en.2008-0070)

Ho K 2011 A critically swift response: insulin-stimulated potassium and glucose transport in Skeletal Muscle. *Clinical Journal of the American Society of Nephrology* **6** 1513–1516. (doi:10.2215/CJN.04540511)

Hong EG, Kim BW, Jung DY, Kim JH, Yu T, Da Silva WS, Friedline RH, Bianco SD, Seslar SP, Wakimoto H, *et al*. 2013 Cardiac expression of human type 2 iodothyronine deiodinase increases glucose metabolism and protects against doxorubicin-induced cardiac dysfunction in male mice. *Endocrinology* **154** 3937–3946. (doi:10.1210/en.2012-2261)

Kim SR, Tull ES, Talbott EO, Vogt MT & Kuller LH 2002 A hypothesis of synergism: the interrelationship of T 3 and insulin to disturbances in metabolic homeostasis. *Medical Hypotheses* **59** 660–666. (doi:10.1016/s0306-9877(02)00211-6)

Klieverik LP, Sauerwein HP, Ackermans MT, Boelen A, Kalsbeek A & Fliers E 2008 Effects of thyrotoxicosis and selective hepatic autonomic denervation on hepatic glucose metabolism in rats. *American Journal of Physiology: Endocrinology and Metabolism* **294** E513–E520. (doi:10.1152/ajpendo.00659.2007)

Klieverik LP, Coomans CP, Endert E, Sauerwein HP, Havekes LM, Voshol PJ, Rensen PCN, Romijn JA, Kalsbeek A & Fliers E 2009 Thyroid hormone effects on whole-body energy homeostasis and tissue-specific fatty acid uptake in vivo. *Endocrinology* **150** 5639–5648. (doi:10.1210/en.2009-0297)

Lei J, Mariash CN & Ingbar DH 2004 3, 3′, 5-Triiodo-l-thyronine up-regulation of Na,K-ATPase activity and cell surface expression in alveolar epithelial cells is Src kinase- and phosphoinositide 3-kinase-dependent. *Journal of Biological Chemistry* **279** 47589–47600. (doi:10.1074/jbc.M405497200)

Lin Y & Sun Z 2011 Thyroid hormone potentiates insulin signaling and attenuates hyperglycemia and insulin resistance in a mouse model of type 2 diabetes. *British Journal of Pharmacology* **162** 597–610. (doi:10.1111/j.1476-5381.2010.01056.x)

Maia AL, Kim BW, Huang SA, Harney JW & Larsen PR 2005 Type 2 iodothyronine deioinase in the major source of plasma T3 in euthyroid humans. *Journal of Clinical Investigation* **115** 1–10. (doi:10.1172/JCI25083.The)

Marsili A, Ramadan W, Harney JW, Mulcahey M, Castroneves LA, Goemann IM, Wajner SM, Huang SA, Zavacki AM, Maia AL, *et al*. 2010 Type 2 iodothyronine deiodinase levels are higher in slow-twitch than fast-twitch mouse skeletal muscle and are increased in hypothyroidism. *Endocrinology* **151** 5952–5960. (doi:10.1210/en.2010-0631)

Marsili A, Aguayo-Mazzucato C, Chen T, Kumar A, Chung M, Lunsford EP, Harney JW, van-Tran T, Gianetti E, Ramadan W, *et al*. 2011 Mice with a targeted deletion of the type 2 deiodinase are insulin resistant and susceptible to diet induced obesity. *PLoS ONE* **6** e20832. (doi:10.1371/journal.pone.0020832)

Martinez B, Sonanez-Organis JG, Viscarra JA, Jaques JT, MacKenzie DS, Crocker DE & Ortiz RM 2016 Glucose delays the insulin-induced increase in thyroid hormone-mediated signaling in adipose of prolong-fasted elephant seal pups. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **310** R502–R512. (doi: 10.1152/ajpregu.00054.2015)

Matsen ME, Thaler JP, Wisse BE, Guyenet SJ, Meek TH, Ogimoto K, Cubelo A, Fischer JD, Kaiyala KJ, Schwartz MW, *et al*. 2013 In uncontrolled diabetes, thyroid hormone and sympathetic activators induce thermogenesis without increasing glucose uptake in brown adipose tissue. *American Journal of Physiology: Endocrinology and Metabolism* **304** E734–E746. (doi:10.1152/ajpendo.00488.2012)

Medina MC, Molina J, Gadea Y, Fachado A, Murillo M, Simovic G, Pileggi A, Hernandez A, Edlund H & Bianco AC 2011 The thyroid hormone-inactivating type III deiodinase is expressed in mouse and human β-cells and its targeted inactivation impairs insulin secretion. *Endocrinology* **152** 3717–3727. (doi:10.1210/en.2011-1210)

Moreno M, Silvestri E, De Matteis R, de Lange P, Lombardi A, Glinni D, Senese R, Cioffi F, Salzano AM, Scaloni A, *et al*. 2011 3,5-Diiodo-l-thyronine prevents high-fat-diet-induced insulin resistance in rat skeletal muscle through metabolic and structural adaptations. *FASEB Journal* **25** 3312–3324. (doi:10.1096/fj.11-181982)

Montez P, Vazquez-Medina JP, Rodriguez R, Thorwald MA, Viscarra JA, Lam L, Peti-Peterdi J, Nakano D, Nishiyama A & Ortiz RM 2012 Angiotensin receptor blockade recovers hepatic UCP2 expression and aconitase and SDH activities and ameliorates hepatic oxidative damage in insulin resistant rats. *Endocrinology* **153** 5746–5759. (doi:10.1210/en.2012-1390)

Moynihan KA, Grimm AA, Plueger MM, Bernal-Mizrachi E, Ford E, Cras-Meneur C, Permutt MA & Imai SI 2005 Increased dosage of mammalian Sir2 in pancreatic β cells enhances glucose-stimulated insulin secretion in mice. *Cell Metabolism* **2** 105–117. (doi:10.1016/j.cmet.2005.07.001)

Müller J, Mayerl S, Visser TJ, Darras VM, Boelen A, Frappart L, Mariotta L, Verrey F & Heuer H 2014 Tissue-specific alterations in thyroid hormone homeostasis in combined Mct10 and Mct8 deficiency. *Endocrinology* **155** 315–325. (doi:10.1210/en.2013-1800)

Rodriguez R, Viscarra JA, Minas JN, Nakano D, Nishiyama A & Ortiz RM 2012 Angiotensin receptor blockade increases pancreatic insulin secretion and decreases glucose intolerance during glucose supplementation in a model of metabolic syndrome. *Endocrinology* **153** 1684–1695. (doi:10.1210/en.2011-1885)

Skarulis MC, Celi FS, Mueller E, Zemskova M, Malek R, Hugendubler L, Cochran C, Solomon J, Chen C & Gorden P 2010 Thyroid hormone induced brown adipose tissue and amelioration of diabetes in a patient with extreme insulin resistance. *Journal of Clinical Endocrinology and Metabolism* **95** 256–262. (doi:10.1210/jc.2009-0543)

Suh JH, Sieglaff DH, Zhang A, Xia X, Cvoro A, Winnier GE & Webb P 2013 SIRT1 is a direct coactivator of thyroid hormone receptor β1 with gene-specific actions. *PLoS ONE* **8** e70097. (doi:10.1371/journal.pone.0070097)

Sun C, Zhang F, Ge X, Yan T, Chen X, Shi X & Zhai Q 2007 SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B. *Cell Metbolism* **6** 307–319. (doi:10.1016/j.cmet.2007.08.014)

Teixeira SS, Tamrakar AK, Goulart-Silva F, Serrano-Nascimento C, Klip A & Nunes MT 2012 Triiodothyronine acutely stimulates glucose transport into L6 muscle cells without increasing surface GLUT4, GLUT1, or GLUT3. *Thyroid* **22** 747–754. (doi:10.1089/thy.2011.0422)

Thakran S, Sharma P, Attia RR, Hori RT, Deng X, Elam MB & Park EA 2013 Role of sirtuin 1 in the regulation of hepatic gene expression by thyroid hormone. *Journal of Biological Chemistry* **288** 807–818. (doi:10.1074/jbc.M112.437970)

Toda C & Diano S 2014 Mitochondrial UCP2 in the central regulation of metabolism. *Best Practice and Research: Clinical Endocrinology and Metabolism* **28** 757–764. (doi:10.1016/j.beem.2014.02.006)

Van Der Deure WM, Peeters RP & Visser TJ 2010 Molecular aspects of thyroid hormone transporters, including MCT8, MCT10, and OATPs, and the effects of genetic variation in these transporters. *Journal of Molecular Endocrinology* **44** 1–11. (doi:10.1677/JME-09-0042)

Vázquez-Medina JP, Popovich I, Thorwald MA, Viscarra JA, Rodriguez R, Sonanez-Organis JG, Lam L, Peti-Peterdi J, Nakano D, Nishiyama A, *et al.* 2013 Angiotensin receptor-mediated oxidative stress is associated with impaired cardiac redox signaling and mitochondrial function in insulin-resistant rats. *American Journal of Physiology: Heart and Circulatory Physiology* **305** H599–H607. (doi:10.1152/ajpheart.00101.2013)

Villicev CM, Freitas FRS, Aoki MS, Taffarel C, Scanlan TS, Moriscot AS, Ribeiro MO, Bianco AC & Gouveia CHA 2007 Thyroid hormone receptor β-specific agonist GC-1 increases energy expenditure and prevents fat-mass accumulation in rats. *Journal of Endocrinology* **193** 21–29. (doi:10.1677/joe.1.07066)

Wang CZ, Wei D, Guan MP & Xue YM 2014 Triiodothyronine regulates distribution of thyroid hormone receptors by activating AMP-activated protein kinase in 3T3-L1 adipocytes and induces uncoupling protein-1 expression. *Molecular and Cellular Biochemistry* **393** 247–254. (doi:10.1007/s11010-014-2067-6).

Weitzel JM, Radtke C & Seitz HJ 2001 Two thyroid hormone-mediated gene expression patterns in vivo identified by cDNA expression arrays in rat. *Nucleic Acids Research* **29** 5148–5155. (doi:10.1093/nar/29.24.5148)

Yuan Q, Zhou QY, Liu D, Yu L, Zhan L, Li XJ, Peng HY, Zhang XL & Yuan XC 2014 Advanced glycation end-products impair Na+/K+-ATPase activity in diabetic cardiomyopathy: role of the adenosine monophosphate-activated protein kinase/sirtuin 1 pathway. *Clinical and Experimental Pharmacology and Physiology* **41** 127–133. (doi:10.1111/1440-1681.12194)