Intramuscular triglyceride content precedes impaired glucose metabolism, without evidence for mitochondrial dysfunction during early development of a diabetic phenotype.

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Intramuscular triglyceride content precedes impaired glucose metabolism, without evidence for mitochondrial dysfunction during early development of a diabetic phenotype.

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Running Title: Muscle lipids in early stages of type 2 diabetes

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ABSTRACT

The incidence of type 2 diabetes is highly correlated with obesity; however, there is a lack of research elucidating the temporal progression. Transgenic FVB/N UCP-dta mice, which develop a diabetic phenotype, and their non-transgenic littermates were fed either a high-fat or normal-chow diet and were studied at 6, 9, 12, 15, 18, 21, and 24 weeks of age in order to test the hypothesis that increased lipid accumulation in skeletal muscle causes mitochondrial dysfunction, leading to the development of insulin resistance. Body composition, intramuscular triglyceride (IMTG) content, glucose metabolism, and mitochondrial function were measured to determine if IMTG drove mitochondrial dysfunction, leading to the development of type 2 diabetes. High-fat fed transgenic mice had a significantly greater body mass, lipid mass, and intramuscular triglyceride (IMTG) content beginning early in the experiment. Glucose tolerance tests revealed that high-fat fed transgenic mice developed a significantly insulin resistant response compared to the other 3 groups toward the end of the time course while plasma insulin was elevated very early in the time course. There was no significant difference in several measures of metabolic function throughout the time course. Long-term high-fat feeding in transgenic mice produced increases in IMTG, adiposity, body mass, and plasma insulin accompanied by decreases in glucose metabolism, but did not reveal any deficits in mitochondrial function or regulation during the early stage of the development of type 2 diabetes. It does not appear that lipotoxicity is driving defects in mitochondrial function prior to the onset of insulin resistance.

Key Words: obesity, diabetes, lipotoxicity, nuclear receptors.

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INTRODUCTION

Obesity is accompanied by increases in both visceral and intracellular lipids. There is evidence for the deleterious effects of visceral lipid accumulation in numerous pathologies (Berkalp et al. 1995, Shimabukuro et al. 1998, Marceau et al. 1999). For example, a negative relationship between excessive intracellular lipids and cardiac myopathy was first described more than 40 years ago (Chu et al. 1969). There is also evidence of a strong negative association between pancreatic lipids and β -cell dysfunction (Unger 1995, Shimabukuro et al. 1998, Tushuizen et al. 2007) further exacerbating glucose metabolism. The association between increased intracellular lipids and metabolic dysfunction led to a proposed theory of ‘lipotoxicity,’ in which increased intracellular lipid accumulation was not merely correlative, but was causative of insulin resistance and other metabolic disorders.

Like other non-adipose tissues, increases in intracellular triglycerides in skeletal muscle (IMTG) are correlated with defects in glucose homeostasis (Simoneau et al. 1995, Simoneau and Kelley 1997, Goodpaster et al. 1997). As skeletal muscle accounts for approximately 70% of whole-body glucose metabolism (Shulman et al. 1990), decrements in the ability of skeletal muscle to maintain glucose homeostasis can have widespread effects in whole-body glucose metabolism. Numerous studies show a negative correlation between insulin sensitivity and IMTG (Kraegen et al. 1991, Phillips et al. 1996, Pan et al. 1997, Russell et al. 1998, Krssak et al. 1999). Recent studies have also shown that increased IMTG are associated with a preference towards lipid oxidation and a reduction in glucose metabolism (Thyfault et al. 2006, Sapiro et al. 2009, Zechner et al. 2009). However, other investigations revealed a paradox to the ‘lipotoxicity’ theory as endurance-trained athletes exhibit similarly high levels of IMTG as obese, diabetic patients without impairment of insulin-stimulated glucose uptake (Goodpaster et al. 2001,

Russell 2004, Dubé et al. 2008). These data suggest that IMTG may be merely correlated with metabolic dysfunction, rather than causative.

In addition to reduced insulin sensitivity, impairment of mitochondrial function in skeletal muscle is reported in diabetic patients (Kelley et al. 2002, Mootha et al. 2003, Patti et al. 2003) and animal models of type 2 diabetes (Boudina et al. 2005, Warren et al. 2014). Obese subjects also demonstrate an inability to transition between lipid and glucose metabolism and have a subsequent increase in lipid oxidation (Goodpaster and Kelley 2002). Recent investigations note several possible drivers of mitochondrial dysfunction in response to intramuscular lipid accumulation, including reduced functional capacity (Ritov et al. 2005) and a reduction in mitochondrial biogenesis (Kelley et al. 2002). However, increases in mitochondrial number are reported in insulin resistant rats fed a high-fat diet (Hancock et al. 2008). So although the correlation between mitochondrial dysfunction and increased intramuscular lipids is clear, the mechanisms by which these factors affect disease states are unresolved (Storlien et al. 2004).

Many of the studies relating obesity with insulin resistance or mitochondrial dysfunction describe relationships in a developed diabetic condition. The temporal effects that high-fat feeding has, and the progressive changes in IMTG content, glucose metabolism, and mitochondrial function that occur during the development of a diabetic phenotype are unclear. The current study was designed using a longitudinal model, which allows for temporal changes in IMTG levels to be contrasted against changes in muscle mitochondrial function and systemic insulin resistance. Our investigations focused on elucidating possible defects in lipid storage/catabolism and mitochondrial function throughout the initial stages of the pathogenesis of type 2 diabetes. The time-course nature of the study will also help determine whether mitochondrial dysfunction drives increases in IMTG or if increases in IMTG drive mitochondrial

dysfunction. As such, this study provides a test of the role of ‘lipotoxicity’ in driving early mitochondrial defects, metabolic dysfunction and/or type 2 diabetes.

To this end, we compared control mice with several models of obesity, both wild-type mice fed a high fat diet, and UCP-dta mice fed normal or high fat diet. The UCP-dta mice are deficient in brown adipose tissue (Lowell et al. 1993), and are known to develop obesity and insulin resistance, especially when fed a high fat diet (Hamann et al. 1995). Our hypothesis is that intracellular skeletal muscle lipid accumulation will precede mitochondrial dysfunction and will drive further perturbations in metabolism, leading to the development of type 2 diabetes.

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MATERIALS AND METHODS

Animals and Experimental Design.

FVB/N UCP-dta mice (Tg) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). These mice express the diphtheria toxin A-chain under control of the uncoupling protein 1 promoter, which largely ablates brown adipose tissue (Lowell et al. 1993), and when fed a 'Western diet,' develop marked obesity, insulin resistance, hyperglycemia, and hyperlipidemia (Hamann et al. 1995). A breeding colony was established at the Miami University Animal Care Facility and non-transgenic littermates were used as controls (Ct). We used only male mice in this study and all mice were housed under conditions of controlled temperature and humidity with a 12:12-h L:D cycle and given water ad libitum. All animal experiments and euthanasia protocols were conducted in accordance with the National Institutes of Health guidelines for humane treatment of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of Miami University.

Upon weaning (3 weeks of age), mice were randomly selected and fed either standard chow diet (Purina Mills # 5001, Ch – 13.5% of calories from lipids) or high-fat diet (Teklad Laboratory Animal diets # TD.06415, HF - 45% of calories from lipids; Harlan Laboratories, Indianapolis, IN, USA). This created 4 different genotype/diet groups: wild-type control chow (WtCh), wild-type high-fat (WtHF), transgenic chow (TgCh), and transgenic high-fat (TgHF). At 6 weeks of age a subset of mice (8-10 per diet/phenotype group) was randomly chosen, longitudinal measurements of body mass and body composition were taken and a glucose tolerance test was performed, all at three week intervals until 24 weeks of age. A second subset of mice (8-10 per diet/phenotype group at each time point) was randomly selected for analysis of intramuscular lipid content, plasma metabolites, gene expression, and citrate

synthase/cytochrome c enzyme activity. The mice from subset 2 were randomly assigned a sacrifice date of 6, 9, 12, 15, 18, 21, or 24 weeks of age and plasma and gastrocnemius muscle were collected and stored at -80°C for procedures as described below.

Body Mass and Composition.

Starting at six weeks of age, mice from subset 1 (n=8-10/group) were weighed twice weekly to generate growth curves. Additionally, lean mass and fat mass were determined via quantitative nuclear magnetic resonance (NMR) using an EchoMRI™ – 500, (Echo Medical Systems, Houston, TX, USA) at 6 weeks of age, and every three weeks thereafter (9, 12, 15, 18, 21, and 24 weeks).

Glucose tolerance testing.

Using the same subset of animals as above, beginning at 6 weeks of age and every three weeks thereafter (9, 12, 15, 18, 21, and 24 weeks), a glucose tolerance test was performed (n=8-10 for each group/time point). After an overnight fast, a blood sample was collected from the tail vein and glucose concentration was measured by the glucose oxidase method with a reflectance glucometer (One Touch II, LifeScan, Milpitas, CA, USA). A 10% glucose solution (1 mg glucose/g body wt) was administered by intraperitoneal injection and subsequent blood samples were obtained 30 min, 60 min and 120 min after injection. Glucose area under the curve (AUC) calculations were performed following (Tai 1994) after baseline (minute 0) plasma glucose concentration was subtracted to eliminate the effect of intra-individual variations.

Intramuscular lipid content determination.

Beginning at 6 weeks of age and every three weeks thereafter (9, 12, 15, 18, 21, or 24 weeks), mice from the second subset were sacrificed and tissues collected (n=8-10/group at each time point). Lipids were extracted from a piece of the gastrocnemius (GC) muscle using a modified Folch method as described (Folch et al. 1957). A piece of the frozen gastrocnemius was weighed and minced in 40ml of 2:1 chloroform:methanol. After 24 hours at 4°C, the sample was filtered, 60ml of 1:1 H₂O:chloroform was added and left overnight to separate via gravity in a separatory funnel. The lower phase was drained and evaporated under nitrogen stream using a NEVAP 111 (Organomation, Berlin, MA, USA). The remaining dry extract was weighed and the lipid content (as a percentage of original tissue mass) was obtained.

Plasma Metabolite Analysis.

At sacrifice, blood was drawn from the inferior vena cava of mice (n= 8-10/group at each time point), into a syringe pre-coated with 0.5M EDTA (pH 8.0) as anti-coagulant. Blood was held on ice for about 30 minutes, then centrifuged at 4,000g for 5 minutes. Supernatant was transferred to a clean microfuge tube, frozen in liquid nitrogen and stored at -80°C. Plasma was sent to the Mouse Metabolic Phenotyping Core at the University of Cincinnati for determination of plasma substrate (free fatty acids, triglyceride, and glucose) concentrations as well as plasma insulin concentrations using standard protocols.

Quantitative RT-PCR.

Using gastrocnemius tissues collected from mice in subset 2 (n= 8-10/group at each time point), quantitative RT-PCR was used to assess expression levels of transcripts for several genes. Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA; Table 1 for

sequences). RNA was isolated from GC muscle using the TRIzol method (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. RNA quality was assessed spectrophotometrically and quantified by absorption spectrophotometry at 260 and 280 nm (NanoDrop 1000, NanoDrop-Fisher Thermo Scientific, Rockford, IL, USA). cDNA was generated from 1 µg of total RNA for each GC muscle using qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's instructions. Relative quantitative RT-PCR was subsequently performed with a RotorGene 3000 (Qiagen, Valencia, CA, USA) system using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). For each gene, real-time PCR was performed in duplicate wells on cDNA generated from the reverse transcription of 10 ng of total RNA. Primer efficiencies were validated and verified for all genes and expression data were normalized to 36B4 (acidic ribosomal phosphoprotein P0), which did not change with treatment. Genes investigated were: PPAR α (Peroxisome proliferator-activated receptor alpha); PPAR β (Peroxisome proliferator-activated receptor beta); PGC-1 α (Peroxisome proliferator-activated receptor gamma coactivator-1alpha); PGC-1 β (Peroxisome proliferator-activated receptor gamma coactivator-1beta); MCAD (Medium chain acyl-CoA dehydrogenase); M-CPT I (Muscle Carnitine palmitoyltransferase I); CD36 (Fatty Acid Translocase); ATP synthase (F₀F₁ complex); CCO (Cytochrome c oxidase); and CS (Citrate synthase).

Citrate synthase and Cytochrome c enzyme activity.

Using gastrocnemius tissues collected from mice in subset 2 (n= 8-10/group at each time point), assays for citrate synthase (CS) and cytochrome c oxidase (CCO) activity were performed following the methods of Berner and Puckett (2010). Citrate synthase activity was determined

from the reduction of DTNB (5,5' dithiobis-2-nitrobenzoic acid) at 412 nm in assay medium containing 100 mM Tris-HCL (pH=8.0), 0.1mM DTNB, 0.15mM acetyl-CoA, 0.15 mM oxaloacetate (omitted for control). CCO activity was determined from the oxidation of reduced cytochrome c at 550 nm against a reference of 0.075 mM cytochrome C oxidized with 0.33% potassium ferricyanide. The CCO assay medium contained 100 mM potassium phosphate (pH=7.5) and 0.075 mM reduced cytochrome c. Cytochrome c was reduced with sodium hydrosulfite, and excess sodium hydrosulfite was removed by bubbling with air for 30 min. All assays were performed in duplicate on a temperature-controlled spectrophotometer (PerkinElmer Lambda 35, Hebron, KY, USA) at 37°C. CS and CCO reactions were followed for 5 minutes. Enzyme activities were calculated from the slope of the linear portion of the reactions. Enzyme activity is expressed as units/gram of wet tissue, where 1 unit is equal to 1 μ mol of product/min.

Statistical analysis.

A univariate split-plot repeated measures analysis of variance (ANOVA) analysis was performed on the data for body mass and composition as well as plasma glucose concentration measurements using JMP statistical software (version 11.0.0) with genotype, diet, and time as factors. Data for the intramuscular lipid content, plasma metabolite analysis, gene expression, and citrate synthase/cytochrome c enzyme activity assay was analyzed using ANOVA with genotype, diet, and time as factors. For full ANOVA output, see supplementary table S1 and supplementary table S2. Pairwise comparisons are reported in the running text of the results section. When significant differences were detected in parameters, pairwise comparisons were run using the Tukey HSD method. The level of significance was set at $p < 0.05$. Data are presented as means $\pm$ SEM (n).

RESULTS

Body Mass and Composition.

To describe the progression of obesity, mice from subset 1 were weighed twice weekly and body composition determined every three weeks. Although we had anticipated intermediate phenotypes in the high fat fed wild types and chow fed transgenic mice, pairwise comparison revealed increased body, fat or lean mass only in the transgenic, high fat fed mice (TgHF). Increased body mass was already evident at 6 weeks of age (after only 3 weeks of HF diet) and continued throughout the experiment. By week 24, the TgHF mice were approximately 90% heavier than mice in the other three groups (Figure 1a). Body composition analysis revealed that the majority of the mass increase was due to increased fat mass, which was also already evident at 6 weeks of age (Figure 1b). By 24 weeks, the TgHF mice had more than double any the fat mass of any other groups and more than 5x that of controls (WtCh). While less vivid, lean mass was also elevated in the TgHF group, beginning at 12 weeks of age (Figure 1c).

Measurement of Intramuscular Lipid Content.

We measured intramuscular lipid content to assess the effects that high-fat feeding and genotype had on IMTG. Mice from subset 2 were sacrificed and IMTG content was determined from frozen gastrocnemius muscle. Similar to body and fat mass, pairwise comparisons revealed that lipid accumulation was only evident in the TgHF mice (Figure 2). IMTG was significantly elevated at 9 weeks of age and maintained at about twice the fat mass of any other groups throughout the study.

Plasma Metabolites.

Plasma was taken from sacrificed animals in order to determine any changes in circulating metabolite levels occurring throughout the time course of the study. Plasma triglycerides (TG) were only affected by diet such that both Wt and Tg mice had lower plasma TG levels when HF fed, compared to the chow fed groups (Table 2). There was also a significant effect of diet on circulating non-esterified fatty acid (NEFA) concentration with high-fat fed Wt and Tg mice having higher levels than chow fed mice (Table 2). Additionally, there was a significant diet $\times$ age interaction, as NEFA levels increased in older HF fed mice (Table 2). Plasma glucose concentrations remained constant throughout the experiment (Table 2).

Plasma Insulin and Glucose Tolerance Testing.

To track the development of insulin resistance and type 2 diabetes, beginning at 6 weeks and continuing throughout the time course of the study plasma was drawn at sacrifice of mice in subset 2 and glucose tolerance tests were performed on mice from subset 1. Pairwise comparisons showed that plasma insulin in the TgHF mice was significantly elevated compared to the other groups, which did not differ from one another (Figure 3a), although the chow fed transgenic group showed increases that approached significance ($p = 0.09$) in the latter time points (weeks 21 and 24). Increased insulin levels in the TgHF group reached significance by 9 weeks of age and remained elevated throughout the remainder of the study (Figure 3a). Note that in the last two time points, the values for the TgHF were all at the detection limit of the assay.

The area under the curve calculated from the glucose tolerance tests (AUC), a measure of the deviation from baseline following a glucose injection, showed that the TgHF mice had significantly worse glucose control at weeks 9, 12, 21 and 24 while it did not reach significance at weeks 15 or 18 (Figure 3b). The effect was an increase of about 35-40% at these points and

did not progress with age. There was no significant difference in AUC among the other three groups at any time point.

Metabolic Enzyme Activity.

To determine if high-fat feeding and/or genotype had an effect on muscle mitochondrial function during the development of type 2 diabetes, we measured enzyme activity of citrate synthase (CS) and cytochrome c oxidase (CCO). The age $\times$ genotype interaction was significant for CS activity, however pairwise comparisons only showed a significant elevation of both 24-week-old transgenic groups compared to either wild-type group at 24 weeks of age (Figure 4a). There were no significant differences seen for CCO enzyme activity at any time point (Figure 4b).

Expression of Metabolic Regulatory Genes and Downstream Metabolic Genes.

To note the effect of the development of type 2 diabetes on metabolic flexibility and control, including fatty acid transport and mitochondrial function, we measured the expression of genes in the PPAR/PGC family of transcriptional regulators as well as several of their target genes. There was no significant effect of any variable on the gene expression for PPAR α , PPAR δ , PGC-1 α , or PGC-1 β (Figure 5a-d). Similarly, there was also no significant effect on expression of genes involved in fatty acid transport: CD36 and MCPT1; fatty acid oxidation: MCAD; or mitochondrial function: CS, Cytochrome c oxidase and ATP Synthase (Figure 6a-f).

DISCUSSION

In this report we describe the effects of the development of obesity on IMTG levels and mitochondrial function through the onset of the pathophysiology of type 2 diabetes. Although studies have noted a correlation between increased IMTG, decreased mitochondrial function and diabetic conditions (Simoneau et al. 1995, Simoneau and Kelley 1997, Goodpaster et al. 1997, Koves et al. 2008) it remains unclear whether mitochondrial dysfunction drives early development of type 2 diabetes. An alternative hypothesis is that mitochondrial defects appear after other alterations occur during disease development. The concept of lipotoxicity proposed that accumulation of lipids in non-adipose tissue drives dysfunction, including mitochondrial, and the death of cells (Unger 1995, Shimabukuro et al. 1998). In contrast, observations from athletes revealed accumulation of IMTG in muscle at levels similar to that of obese, type 2 diabetics, with normal metabolic function preserved (Goodpaster et al. 2001). This “athlete’s paradox” suggests that it is not the mere presence of IMTG that is causative of metabolic dysfunction and demonstrate the clear need for research investigating the temporal relationship between metabolic substrate selection and mitochondrial stress (Muoio 2010), particularly in the pathogenesis of type 2 diabetes, as skeletal muscle is responsible for a majority of glucose metabolism. Perturbations in muscle lipid content or metabolic function that impact muscle glucose uptake have severe effects on whole-body glucose homeostasis.

Our results indicate that increases in IMTG, increases in plasma insulin, and the beginning of defects in glucose uptake precede defects in function and transcriptional regulation of mitochondria. Consistent with previous findings, which described a large gain in body mass in mice fed a high-fat diet for 24 weeks (Weisberg et al. 2006), we noted a 66% difference in body mass of transgenic mice fed a high-fat diet for 18 weeks as compared to standard-chow fed transgenic or wild-type mice. Although our body composition data revealed that TgHF mice had

a slightly higher lean mass than other groups, the TgHF mice had up to nearly 400% more fat mass than other groups. Neither the TgCh mice nor the control high-fat fed mice exhibited any difference in lipid content over the time course of the experiment. The effects of the high-fat feeding were also seen in the large difference in lipid content of the gastrocnemius muscle. By 9-weeks of age, TgHF mice had almost double the intramuscular lipid content of the other mice, which is maintained throughout the rest of the study. These results are similar to other transgenic murine studies, which show large increases in IMTG when fed high-fat food (Costford et al. 2006, Liu et al. 2007).

The large difference in overall fat mass and IMTG content noted between TgHF and the other groups was not accompanied by increases in plasma triglycerides. High fat diet led to slightly lower levels of plasma triglycerides and slightly higher plasma NEFA, which could be explained by an increased reliance on β -oxidation (Hancock et al. 2008, Kraegen et al. 2008, Noland et al. 2009) and metabolic inflexibility (Noland et al. 2009, van Herpen et al. 2011) reported previously in diabetic models. Although plasma triglycerides and NEFA levels were similar between both high-fat groups, plasma insulin levels in TgHF mice were ~500% greater. Further evidence to support the reliance upon lipid metabolism and metabolic inflexibility can be seen in the results of the glucose tolerance test, which showed that at 24 weeks the TgHF mice had a 36% larger area under the curve, which together with the elevated plasma insulin, indicated that the TgHF mice were beginning to exhibit failure of insulin stimulated glucose uptake. Clearly, diet altered blood metabolites, but only in the transgenic mice do we see the connection between increased plasma NEFA and increased visceral and intramuscular adiposity. We were surprised to note essentially no difference in the obesity phenotype of the WtHF or TgCh groups. Previous work has found at least mild obesity in these treatments. It may be that the UCP-dta

mice have undergone selection or genomic modification, reducing the severity of the phenotype (Zhou et al. 2014). Further, the wild-type high fat fed mice may not have shown a response as the 45% fat composition is lower than the 58% fat that is used by some groups (King 2012).

Although it has been reported previously that mice fed high-fat chow for as little as two-weeks have decreased mitochondrial function (Sadler et al. 2012), we noted neither changes in gene expression for CS, CCO, ATP Synthase, MCAD, CD36, or M-CPT I, nor reduction of CS or CCO enzyme activity. Thus although IMTG content in TgHF mice was elevated, even in the early stages of the experiment, there was no evidence of any mitochondrial defect. That intramuscular lipid accumulation and insulin resistance were observed leads us to conclude that mitochondrial dysfunction does not appear to drive the development of type 2 diabetes. This data supports findings which show that not only does high-fat feeding have little effect on mitochondrial function in rodents, there have been reports of upregulation of mitochondrial enzymes and mitochondrial transcriptional factors (Koves et al. 2005, Noland et al. 2007, Turner et al. 2007, Hancock et al. 2008). The CS activity in the Tg groups began to be elevated in the final time point, further supporting the idea that mitochondrial dysfunction is not driving early development of type 2 diabetes. Further, we recently showed that mitochondrial function in the UCP-dta mice at baseline is not different from wild-type (Mineo et al., 2012). These results are also similar to those reported previously in human athletes, which saw no difference in insulin sensitivity in endurance trained individuals with high IMTG levels and enhanced mitochondrial function (Goodpaster et al. 2001). Thus the mere presence of IMTG is not directly causative for a decrease in insulin sensitivity, supporting the idea of a paradox between pathological and physiological high levels of IMTG and the idea that IMTG in athletes are physiological while those in obesity are pathological.

We also saw no evidence for a decrease in the expression of transcriptional regulators of mitochondrial function, as expression of PPAR α , PPAR β , and PGC-1 α and PGC-1 β were unchanged throughout the pathogenesis of type 2 diabetes. Similarly, in humans it has been reported that insulin-resistant offspring of type 2 diabetic parents showed reduced mitochondrial density without changes in PGC-1 α or PGC-1 β (Morino et al. 2005). Therefore, it does not appear that mitochondrial dysfunction is a precursor of increased IMTG content or defects in glucose metabolism.

Although there is a strong correlation between obesity and type 2 diabetes, temporal studies are beginning to reveal the role of lipids in the development of the disease. Our data are in agreement with two studies that also found a lack of mitochondrial defect during the pathogenesis of type 2 diabetes using NMR spectroscopy in rats (De Feyter et al. 2008) and using electron microscopy, respiration in isolated fibers and gene expression in mice (Bonnard et al. 2008). There is considerable evidence that lipid accumulation may have ongoing effects that include mitochondrial damage in later stages of the disease development (Abdul-Ghani et al. 2008, Bonnard et al. 2008, Koves et al. 2008). Thus obesity prevention should remain an important intervention. These results help describe the early temporal phenotypic changes in glucose sensitivity and clearly demonstrate that decrements in mitochondrial function are not causative at this stage of disease development. The projected growth of the type 2 diabetes epidemic clearly requires continued investigation into the etiology of the disease to identify early markers for either lifestyle changes or pharmaceutical intervention. Previous work has found that subsarcolemmal fat accumulation is associated with type 2 diabetes (Nielsen et al. 2010) and that the composition of lipids in the muscle may influence insulin sensitivity (Bergman et al. 2012).

We have characterized aspects of the early stages in the development of type 2 diabetes. We found that increased adiposity occurred prior to the appearance of any evidence for insulin resistance. Further, as insulin resistance began to be evident, there was no evidence for mitochondrial dysfunction. Thus we reject the hypothesis that mitochondrial dysfunction is a causative agent in early development of type 2 diabetes. Potentially important, the UCP-dta mice may have been hyperphagic or less active, leading in part to the observed phenotypes, however we did not measure either parameter. The relative importance of feeding and activity should be investigated as potential contributors to the early development of both adiposity and type 2 diabetes. Further, we were unable to directly address the relevance of different lipid species. Absent this information, we nonetheless observed that the accumulation of intracellular fats can directly impact type 2 diabetes development in the absence of any mitochondrial defects. The mechanistic nature of a causative relationship awaits further study, however our results support rejection of a role for mitochondrial dysfunction as a primary cause of type 2 diabetes.

CONFLICTS OF INTEREST

The authors report no conflicts of interest associated with this manuscript.

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Table 1 Primer sequences for qRT-PCR.

Gene	Forward	Reverse
PPAR α	ACTACGGAGTTCACGCATGT	TTGTCGTCAACCAGCTTCAGC
PPAR β	TCACCGGCAAGTCCAGCC	ACACCAGGCCCTTCTCTGGCT
PGC-1 α	CGGAAATCATATCCAACCAG	TGAGGACCACTAGCAAGTTTG
PGC-1 β	TCCAGAAGTCAGCGGCCT	CTGAGCCCGCAGTGTGG
MCAD	GGCTCCTGAGAAGTGTTTCT	GGCTCCTGGTTGTGAGC
M-CPT I	TCTAGGGAATGCCGTTTAC	GAGCACATGGGCACCATAC
CD36	CAAGCTCCTTGGCATGGTAG	TGGATTTGCAAGCACAATATG
F ₀ F ₁ -ATPase	CGTGAGGGCAATGATTTATA	TCCTGGTCTCTGAAGTATTCA
CCO	ACCAAATCTCCACGGTCTGT	GGATTCTCCAAATACTCCATC
CS	CAAGCAACATGGGAAGA	GTCAGGAAGAACCGAAGTCT
36B4	AGATGCAGCAGATCCGCAT	ATATGAGGCAGCAGTTTCTCC

Table 2. Values for plasma concentration of selected metabolites. Data are presented as Mean (SEM) and experiment-wide n = 142. * = significant effect of diet, † = significant age x diet effect. There were no significant pairwise comparisons. NEFA=Non-Esterified Fatty Acids.

	6 Wks.	9 Wks.	12 Wks.	15 Wks.	18 Wks.	21 Wks.	24 Wks.
TRIGLYCERIDE (mg/dL)							
WtCh	160.01 (21.04)	174.75 (42.79)	201.07 (34.18)	200.21 (41.63)	169.59 (18.39)	146.19 (19.24)	201.45 (15.53)
WtHF*	107.05 (18.37)	133.17 (17.79)	108.47 (12.75)	103.28 (17.02)	123.97 (26.32)	110.28 (17.01)	151.23 (27.85)
TgCh	153.25 (18.42)	167.70 (26.66)	168.64 (26.15)	194.11 (32.85)	193.34 (24.82)	173.96 (18.62)	191.70 (36.64)
TgHF*	111.17 (18.53)	110.95 (12.56)	170.62 (18.79)	121.00 (6.55)	124.03 (8.26)	209.05 (62.64)	163.81 (53.53)
NEFA[†] (mEq/L)							
WtCh	0.403 (0.023)	0.373 (0.021)	0.335 (0.023)	0.320 (0.025)	0.363 (0.019)	0.320 (0.020)	0.370 (0.027)
WtHF*	0.074 (0.031)	0.060 (0.055)	0.063 (0.029)	0.060 (0.035)	0.067 (0.031)	0.039 (0.033)	0.047 (0.027)
TgCh	0.340 (0.030)	0.325 (0.024)	0.340 (0.026)	0.319 (0.025)	0.335 (0.027)	0.0336 (0.016)	0.328 (0.019)
TgHF*	0.030 (0.027)	0.024 (0.027)	0.026 (0.022)	0.025 (0.027)	0.027 (0.023)	0.016 (0.069)	0.019 (0.056)
GLUCOSE (mg/dL)							
WtCh	249.26 (16.18)	265.27 (15.75)	253.65 (6.68)	255.03 (10.09)	225.19 (15.66)	218.19 (11.32)	227.92 (25.50)
WtHF	342.2 (36.07)	235.74 (25.45)	260.81 (24.21)	237.61 (27.55)	274.68 (59.55)	250.64 (33.09)	281.31 (57.83)
TgCh	227.62 (19.14)	244.87 (13.11)	228.24 (10.51)	283.29 (32.93)	357.63 (41.66)	276.56 (44.49)	314.47 (44.04)
TgHF	289.07 (23.84)	308.61 (55.93)	298.09 (54.16)	273.55 (38.55)	251.46 (32.59)	302.29 (69.99)	330.24 (46.04)

FIGURE LEGENDS

Figure 1. High-fat diet leads to increases in body mass and adiposity in transgenic mice. Mice ($n = 8-10/\text{group}$) were weighed twice weekly. Body composition was determined via NMR every three weeks ($n = 8-10/\text{group}$). a) Only the TgHF mice increased body mass. * indicates significant pairwise differences between TgHF and the other groups ($p < 0.05$) at every time point. Data revealed that both lean and fat mass increases contributed to the body mass increase, although fat mass increases were greater than lean. b) Fat mass c) Lean tissue mass. In both cases, * indicates significant pairwise differences between TgHF and the other groups ($p < 0.05$) at every (fat) or at indicated (lean) time points. Values are means $\pm$ SEM.

Figure 2. Transgenic mice fed high-fat chow have higher intramuscular lipid content. Intramuscular lipid content of the gastrocnemius muscle ($n = 8-10$ per group and time point) was measured via the Folch method. * indicates significant pairwise differences between TgHF and the other groups ($p < 0.05$). Values are means $\pm$ SEM.

Figure 3. a) Plasma insulin concentration (ng/mL) is higher in transgenic mice fed high-fat chow. Blood was drawn from the inferior vena cava following sacrifice ($n = 8-10$ per group and time point) and plasma insulin concentration was measured. * indicates significant pairwise differences between TgHF and the other groups ($p < 0.05$). There are no error bars for TgHF mice at 21 and 24 weeks because all samples equaled or exceeded the maximum assay value of 110 ng/mL. b) Glucose area under the curve (AUC) is higher in TgHF mice indicating the beginning of insulin resistance. Glucose tolerance tests were performed every three weeks ($n =$

8-10 per group and time point). Baseline (minute 0) plasma glucose levels were subtracted for each animal to eliminate baseline variability in determining the extent of response. * indicates significant pairwise differences between TgHF and the other groups ($p < 0.05$). Values are means $\pm$ SEM.

Figure 4. Activity of two mitochondrial enzymes in homogenates of gastrocnemius muscle ($n = 8-10$ per group and time point) remained nearly unchanged throughout the experimental time course. a) CS enzyme activity. Citrate synthase activity was unchanged except at 24 weeks. * indicates significant pairwise differences between both Tg vs. either Wt group ($p < 0.05$). b) CCO enzyme activity, Cytochrome c oxidase activity was unchanged at any time point. Values are means $\pm$ SEM.

Figure 5. mRNA expression of members of the PPAR/PGC family of transcriptional activators is unchanged in all groups ($n = 8-10$ per group and time point) throughout the time course of the study. a) PPAR α , b) PPAR δ , c) PGC-1 α , d) PGC-1 β . Quantitative expression data for each gene were normalized and corrected for the expression of a housekeeping gene (36B4). There were no significant differences for any target. Values are means $\pm$ SEM.

Figure 6. mRNA expression is not statistically different for genes involved in fatty acid transport and mitochondrial function in all groups ($n = 8-10$ per group and time point) throughout the time course of the study. a) CD36; b) M-CPT I; c) MCAD; d) CS; e) CCO; f) ATP synthase. Quantitative data for each gene were normalized and corrected for the expression of a

housekeeping gene (36B4). There were no significant differences for any target. Values are means $\pm$ SEM.

Draft

Figure 3b.

Figure 5b.

Figure 5d.