Treatment with the 3-ketoacyl-CoA thiolase inhibitor trimetazidine does not exacerbate whole body insulin resistance in obese mice


Cardiovascular Research Centre, Mazankowski Alberta Heart Institute, University of Alberta – JRU, WK, NF, JM, LZ, DGL, CSW, JSJ, GDL
Sarah W. Stedman Nutrition and Metabolism Center – TRK, ORI, DMM
Department of Medicine, Duke University – TRK, ORI, DMM
Department of Pharmacology and Cancer Biology, Duke University – DMM
Running Title: Trimetazidine and Insulin Resistance

Address for correspondence:
Dr. Gary Lopaschuk,
423 Heritage Medical Research Center
University of Alberta
Edmonton, Canada
T6G 2S2

tel: (780) 492-2170
fax: (780) 492-9753
email: gary.lopaschuk@ualberta.ca

Word Count: 6,586
Text Pages: 30
References: 40
Figures: 6
Tables: 1

List of Abbreviations:

3-KAT – 3-ketoacyl CoA thiolase
ANOVA – analysis of variance
CPT-1 – carnitine palmitoyl transferase 1
DAG – diacylglycerol
DMEM – Dulbecco’s modified eagle’s medium
FBS – fetal bovine serum
HFD – high fat diet
HS – horse serum
i.p. - intraperitoneal
LV – left ventricular
RER – respiratory exchange ratio
T2D – type 2 diabetes
TAG – triacylglycerol
ABSTRACT

There is a growing need to understand the underlying mechanisms involved in the progression of cardiovascular disease during obesity and diabetes. While inhibition of fatty acid oxidation has been proposed as a novel approach to treat ischemic heart disease and heart failure, reduced muscle fatty acid oxidation rates may contribute to the development of obesity-associated insulin resistance. Our aim was to determine whether treatment with the antianginal agent, trimetazidine, which inhibits fatty acid oxidation in the heart secondary to inhibition of 3-ketoacyl CoA thiolase (3-KAT), may have off-target effects on glycemic control in obesity. We fed C57BL/6 mice a high fat diet (HFD) for 10 weeks prior to a 22-day treatment with the 3-KAT inhibitor, trimetazidine (15 mg/kg/day). Insulin resistance was assessed via glucose/insulin tolerance testing, while lipid metabolite content was assessed in gastrocnemius muscle. Trimetazidine-treatment led to a mild shift in substrate preference towards carbohydrates as an oxidative fuel source in obese mice, evidenced by an increase in the respiratory exchange ratio. This shift in metabolism was accompanied by an accumulation of long-chain acyl CoA and a trend to an increase in triacylglycerol content in gastrocnemius muscle, but did not exacerbate HFD-induced insulin resistance compared to control treated mice. Interestingly, trimetazidine treatment reduced palmitate oxidation rates in the isolated working mouse heart and neonatal cardiomyocytes, but not C2C12 skeletal myotubes. Our findings demonstrate that trimetazidine therapy does not adversely affect HFD-induced insulin resistance, suggesting that treatment with trimetazidine would not worsen glycemic control in obese patients with angina.
INTRODUCTION

Ischemic heart disease is a major cause of death and disability in the world today. However, results from numerous epidemiological studies and randomized, placebo-controlled trials have provided compelling evidence that ischemic heart disease is highly manageable. Current treatment regimens consist of either percutaneous or surgical techniques to restore myocardial blood and oxygen supply, or pharmacotherapy (i.e. β-adrenergic receptor blockers) to reduce myocardial oxygen demand, and have significantly improved the overall prognosis of patients with angina and/or ischemic heart disease (Anderson et al., 2013). Yet, there remains a significant number of patients who are refractory to conventional treatment, and thus novel therapies to treat ischemic heart disease are necessary. One potential exciting new therapy involves the optimization of cardiac energy metabolism, which can be achieved via reducing myocardial fatty acid oxidation rates (Ussher and Lopaschuk, 2008; Jaswal et al., 2011; Ussher et al., 2012a).

Indeed, pre-clinical studies demonstrate that reducing fatty acid oxidation rates in the heart, either secondary to limiting the mitochondrial uptake of fatty acids, or directly inhibiting the mitochondrial β-oxidation enzymatic machinery, reduces infarct size and improves cardiac function in experimental models of ischemia/reperfusion injury (Kantor et al., 2000; Dyck et al., 2004; Ussher et al., 2012b). Similar findings have been recapitulated in humans, as treatment with either perhexiline, which restricts mitochondrial fatty acid uptake via inhibition of carnitine palmitoyl transferase-1, or trimetazidine, which directly inhibits fatty acid oxidation via inhibiting the mitochondrial β-oxidation enzyme, long-chain 3-ketoacyl CoA thiolase (3-KAT), improves left ventricular (LV) function in ischemic heart failure patients (Lee et al., 2005; Fragnasso et al., 2006; Tuunanen et al., 2008).
Of the metabolic agents available that act via reducing fatty acid oxidation rates, trimetazidine is the best characterized (Kantor et al., 2000; Lopaschuk et al., 2003), and is used clinically in over 80 countries as a treatment for angina (Ciapponi et al., 2005; Ussher and Lopaschuk, 2006). Although reducing fatty acid oxidation rates in the heart may produce beneficial anti-ischemic effects, it has been demonstrated in muscle that decreased fatty acid oxidation rates may promote insulin resistance in the setting of obesity (Choi et al., 2007; Savage et al., 2007). During obesity, excessive fatty acid uptake outpaces mitochondrial oxidative capacity, and as esterified fatty acids are diverted away from carnitine palmitoyl transferase 1 (CPT-1), the rate limiting enzyme in mitochondrial fatty acid uptake, triacylglycerol (TAG) and other lipid metabolites such as ceramide and diacylglycerol (DAG) accumulate, which may have direct negative effects on muscle insulin sensitivity (Shulman, 2000; Chavez and Summers, 2012; Muoio and Neufer, 2012). Thus, it has been proposed that enhancing muscle fatty acid oxidation can protect against insulin resistance by preventing the accumulation of these lipid metabolites (Choi et al., 2007).

Due to these contrasting views regarding fatty acid oxidation rates in heart and muscle, our objective was to determine whether treatment with trimetazidine would exacerbate HFD-induced insulin resistance. Because patients with angina and ischemic heart disease are also often obese and at risk of type 2 diabetes (T2D), it is essential to determine whether trimetazidine may have off-target effects on insulin sensitivity, potentially limiting its therapeutic utility.

METHODS
Animal Studies

All animals received care according to the Canadian Council on Animal Care, and all animal procedures were approved by the University of Alberta Health Sciences Animal Welfare Committee. 26 week-old C57BL/6NCrl mice (Charles River) received a high fat diet (HFD, 60% kcal from lard, Research Diets D12492) for 10 weeks. At the end of week 10, animals were administered trimetazidine hydrochloride (15 mg/kg/day, Sigma 653322) or saline by intraperitoneal (i.p.) injection for 3 weeks (see Figure 1 for study protocol in obese mice). At study completion, ad libitum animals were killed for tissue extraction via i.p. injection of sodium pentobarbital (12 mg) 2 hrs into their dark cycle.

Glucose and Insulin Tolerance

i.p. glucose and insulin tolerance tests were performed 6 hr after food withdrawal using glucose and insulin doses of 2 g/kg and 0.7 U/kg, respectively. Blood glucose levels were determined at 0, 20, 30, 60, and 90 min post-glucose/insulin administration via tail bleed with the Accu Check Advantage system (Roche®).

Plasma Insulin Levels

Plasma insulin concentrations were determined via use of a commercially available enzymatic assay kit (Alpco Diagnostics) as previously described (Bates et al., 2012). In brief, 5 μL of sample was added per well with 75 μL of a provided enzyme conjugate, and the 96 well plate was then incubated for 2 hr at room temperature on an orbital microplate shaker. After incubation, the plate was washed 6x with wash buffer, and then 100 μL of a provided substrate was added to each well to start the reaction, which was terminated after 30 min via addition of
100 µL of stop solution. Air bubbles were removed and plasma insulin levels “ng/mL” were determined via reading the absorbance of the plate at a 450 nm wavelength.

**Isolated Working Heart Perfuions**

Mice were anaesthetized with sodium pentobarbital (60 mg/kg i.p.), and the hearts were subsequently excised for perfusion in the isolated working heart mode as previously described (Ussher et al., 2012b). In brief, hearts were perfused with oxygenated Krebs-Henseleit solution consisting of 5.0 mM glucose, 0.4 mM palmitate bound to 3% fatty acid free bovine serum albumin, and 100 µU/mL insulin. The perfusate was labeled with [U-14C]glucose and [9,10-3H]palmitate, and 3H2O and 14CO2 production were assessed for the measurement of glucose and palmitate oxidation as previously described (Ussher et al., 2012b).

**Muscle Metabolic Profiling**

Gastrocnemius muscle and liver extracts for quantification of acylcarnitine (mass spectrometry/gas chromatography), long-chain acyl CoA (high performance liquid chromatography), TAG (chloroform:methanol extraction), DAG (DAG kinase thin layer chromatography assay), and ceramide (DAG kinase thin layer chromatography assay) content were determined as previously described (Ussher et al., 2010).

**Indirect Calorimetry**

*In vivo* whole body metabolic assessment was performed using an Oxymax Comprehensive Lab Animal Monitoring System (Columbus Instruments) to determine the respiratory exchange ratio, oxygen consumption rates, heat production, locomotor activity, and 24 hr food intake as
previously described (Ussher et al., 2010). Animals were initially acclimatized in the system for a 24 hr period, the subsequent 24 hr period was utilized for data collection.

**Exercise Capacity**

Exercise capacity was performed by running mice on a calibrated, motor-driven treadmill (Columbus Instruments) at a speed of 3 m/min for 1 min, followed by increasing speeds of 4 m/min for 1 min, 5 m/min for 1 min, 6 m/min for 3 min, 8 m/min for 14 min, 9 m/min for 10 min, 10 m/min for 7 min, 12 m/min for 7 min, and 14 m/min until exhaustion. The first 6 min were used as an acclimatization period for the animals to become familiar with the treadmill and not used for data collection. Exhaustion was determined as the animal spending >5 consecutive seconds on the shock grid, or the animal running off the shock grid and immediately falling back onto the shock grid 3 consecutive times.

**Cell Culture**

Primary rat cardiomyocytes were isolated from the hearts of 1- to 3-day old neonatal rat pups (Biosciences) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s Nutrient Mixture F-12 containing 5% fetal bovine serum (FBS), 10% horse serum (HS), and 1% penicillin-streptomycin as previously described (Samokhvalov et al., 2012). C2C12 myotubes (ATCC® CRL-1772) were cultured as myoblasts on Primeria 6 well-plates (Falcon) with DMEM containing 10% FBS and 1% penicillin-streptomycin, while myotube differentiation was induced via culturing in DMEM containing 2% HS and 1% penicillin-streptomycin as previously described (Ussher et al., 2009). Muscle biopsies from vastus lateralis of lean women were extracted via the percutaneous needle biopsy technique, and cultured myoblasts from the
biopsy were subsequently differentiated into skeletal myocytes as previously described (Kovalik et al., 2011). For glucose and palmitate oxidation experiments, media was switched to Krebs-Henseleit solution consisting of 11.0 mM glucose and 0.8 mM palmitate bound to 4% fatty acid free bovine serum albumin. The Krebs-Henseleit solution was labeled with either [U-$^{14}$C]glucose or [1-$^{14}$C]palmitate, and $^{14}$CO$_2$ was captured in hyamine hydroxide soaked filter paper for the measurement of glucose and palmitate oxidation in separate experiments as previously described (Samokhvalov et al., 2012). Oleate oxidation in human skeletal myocytes was measured as previously described (Kovalik et al., 2011).

**Statistical Analysis**

The significance of differences between 2-groups was determined by the use of an unpaired, two-tailed Student’s $t$-test. The significance of differences for multiple comparisons was estimated by two-way analysis of variance (ANOVA). When ANOVA revealed differences, multiple t-tests with a Bonferroni correction were performed on the data sets. Differences were considered significant when $P < 0.05$.

**RESULTS**

**Trimetazidine treatment mildly affects substrate preference in vivo without affecting body weight, adiposity or glycemia in HFD-induced obese mice:** As expected, mice fed a HFD for 10 weeks experienced a significant increase in weight gain (Figure 2A) and became glucose intolerant (Figure 2B). Starting at week 11, animals received daily injections of either trimetazidine (15 mg/kg) or saline for 22 days. Body weight was not altered in trimetazidine-
treated HFD-induced obese mice after 1 week (Figure 2C) or after study completion in both obese (44.85 ± 1.31 g vs. 43.77 ± 1.16 g) and lean mice (28.68 ± 0.99 g vs. 27.64 ± 0.35 g). Likewise, overall adiposity as determined by measurement of epididymal and perirenal adipose depot weights was unaffected in trimetazidine treated HFD-induced obese mice after animal sacrifice at 22 days post-treatment (Figure 2D/E). Furthermore, plasma TAG and free fatty acid levels were similar in trimetazidine treated HFD-induced obese mice following a 6 hr fast at day 21 post-treatment, whereas plasma TAG levels were elevated with no change in free fatty acid levels ad libitum at day 22 post-treatment (Table 1 and Figure 2F/G). At days 16 and 17 post-treatment, indirect calorimetry was assessed through use of metabolic cages, whereby saline treated HFD-induced obese mice exhibited a respiratory exchange ratio (RER) approaching 0.7, indicative of fatty acid oxidation as their primary metabolic substrate. Interestingly, treatment of HFD-induced obese mice with trimetazidine induced a mild shift in metabolic substrate preference, as seen by an increase in RER during the light cycle (Figure 2H/I), illustrating a greater reliance on carbohydrates for oxidative energy metabolism. This shift in metabolism in trimetazidine-treated HFD-induced obese mice was not associated with alterations in whole body oxygen consumption rates, heat production, locomotor activity (Figure 2J-L), or exercise capacity (14.09 ± 2.86 min vs. 14.70 ± 3.11 min during treadmill running) and food intake (2.42 ± 0.17 g vs. 2.35 ± 0.19 g over 24 hrs). Moreover, this mild effect on substrate preference following trimetazidine treatment did not worsen insulin resistance, as glucose tolerance at both 7- and 21-days post-treatment was similar between saline- and trimetazidine-treated HFD-induced obese mice (Figure 3A/B), as were plasma insulin levels during the glucose tolerance test (data not shown). Similarly, insulin tolerance at
14-days post-treatment was also comparable between saline and trimetazidine-treated HFD-induced obese mice (Figure 3C/D).

Trimetazidine inhibits fatty acid oxidation in cardiac but not skeletal muscle: Palmitate oxidation was significantly inhibited in trimetazidine-treated (100 μM) isolated working mouse hearts, which was associated with a corresponding increase in glucose oxidation (Figure 4A). Similar findings were observed in cultured neonatal rat cardiomyocytes (10 μM trimetazidine, Figure 4B). In contrast, trimetazidine (10 μM) did not inhibit palmitate oxidation in C2C12 skeletal muscle myotubes (Figure 4C). Interestingly, however, trimetazidine did stimulate glucose oxidation in C2C12 myotubes (Figure 4C), similar to its effect in the heart (Figure 4A). In addition, we observed a significant decrease in oleate oxidation in primary cultured human myocytes treated with the CPT-1 inhibitor, oxfenicine, but not in response to treatment with trimetazidine (Figure 4D/E).

Muscle and hepatic lipid metabolite content in HFD-induced obese mice after 22 days of treatment with trimetazidine: Treatment with trimetazidine for 22 days resulted in a trend to an increase in gastrocnemius TAG content, which was accompanied by a significant increase in gastrocnemius long-chain acyl CoA content (Figure 5A/B). In contrast, trimetazidine treatment had no effect on gastrocnemius DAG and ceramide content (Figure 5C/D). Gas chromatography/mass spectrometry metabolic profiling of gastrocnemius muscle from control and trimetazidine-treated HFD-induced obese mice demonstrated no change in any of the intermediates of the tricarboxylic acid cycle (Figure 5E). Although the majority of long-chain acylcarnitine species such as palmitoyl- and stearoylcarnitine did not accumulate further in
gastrocnemius muscle of HFD-induced obese mice treated with trimetazidine (*data not shown*), there was a trend to a further increase in other long-chain acylcarnitine species such as palmitoleoylcarnitine (Figure 5F/G). Furthermore, a number of plasma long-chain acylcarnitine species were increased in trimetazidine treated HFD-induced obese mice, consistent with trimetazidine inhibiting muscle fatty acid oxidation *in vivo* (Figure 5H/I). On the contrary, we observed no differences in hepatic TAG, long chain acyl CoA, and ceramide content in HFD-induced obese mice treated with trimetazidine for 22 days (Figure 6).

**DISCUSSION**

This study demonstrates that treatment with the 3-KAT inhibitor, trimetazidine, does not exacerbate HFD-induced insulin resistance, even though trimetazidine treatment resulted in a greater accumulation of intramuscular TAG and long-chain acyl CoA. As previous findings have suggested that a reduction in skeletal muscle fatty acid oxidation can cause insulin resistance and T2D (Choi et al., 2007; Savage et al., 2007), our goal was to determine whether treatment with trimetazidine might have off-target adverse effects on muscle insulin resistance and glycemic control. Surprisingly, while trimetazidine inhibited fatty acid oxidation rates in the isolated working mouse heart and cultured neonatal cardiac myocytes, trimetazidine did not inhibit fatty acid oxidation rates in cultured muscle myotubes, which may explain why trimetazidine did not worsen HFD-induced insulin resistance. However, our *in vivo* indirect calorimetry findings suggest a mild inhibition of whole-body fatty acid oxidation rates, evidenced by the small increase in RER following treatment with trimetazidine, consistent with the increase in intramuscular lipid accumulation. While indirect calorimetry reflects whole
body metabolism, a significant component of the RER is accounted for by skeletal muscle oxidative metabolism (Zurlo et al., 1990; Sleigh et al., 2011).

A potential limitation that may account for the discrepancy between our *in vivo* and *in vitro* findings involves the inherent low oxidative rates measured in the *in vitro* cell culture systems. Indeed, nmol/min fatty acid oxidation rates are often reported *in vivo*/*ex vivo* (Ussher et al., 2012b), versus pmol/min rates *in vitro* (An et al., 2006; Watt et al., 2006). This is probably a reflection of the low work performed by these isolated cell systems, which is an important determinant of mitochondrial oxidative rates (Neely et al., 1967). Indeed, our palmitate oxidation rates measured in both neonatal rat cardiomyocytes and C2C12 myotubes are substantially lower than the rates obtained in our isolated working mouse hearts, which may increase the difficulty of capturing an actual inhibition of fatty acid oxidation. Furthermore, we have previously shown that the ability to measure inhibition of fatty acid oxidation via trimetazidine *in vitro* is complicated by the accumulation of substrate for 3-KAT over time, which overcomes trimetazidine-mediated inhibition of 3-KAT (Lopaschuk et al., 2003). Whether the kinetics for trimetazidine-mediated inhibition of 3-KAT are different in neonatal cardiomyocytes and C2C12 myotubes is of interest, but is beyond the scope of this study’s objectives.

To support our indirect calorimetry observations, we used targeted metabolomics to quantify gastrocnemius and plasma acylcarnitines as an index of potential alterations in fatty acid oxidation. With trimetazidine-mediated inhibition of 3-KAT, the last enzyme involved in mitochondrial fatty acid oxidation, fatty acids would still have free access into the mitochondria through CPT-1, thereby allowing partial oxidation. This is evidenced by similar levels of long-chain acylcarnitines in gastrocnemius muscle from both vehicle control and trimetazidine
treated animals. As skeletal muscle has been demonstrated to be a major source of circulating acylcarnitines (Noland et al., 2009), measurement of plasma acylcarnitines should also provide an index of intramuscular acylcarnitine accumulation and perturbations in fatty acid flux. Indeed, plasma long-chain acylcarnitines were elevated in mice following treatment with trimetazidine, consistent with 3-KAT inhibition not impeding CPT-1-mediated mitochondrial fatty acid uptake, enhancing the accumulation of intramuscular long-chain acylcarnitines and their subsequent export into the circulation.

Therefore, based off on our in vivo observations, we do believe that treatment with trimetazidine results in a very mild inhibition of muscle fatty acid oxidation, which is not captured in our in vitro studies likely due to lack of sensitivity. To our surprise however, this does not result in a worsening of HFD-induced insulin resistance. Although current dogma demonstrates that an acceleration of muscle fatty acid oxidation alleviates insulin resistance via reducing lipid metabolite accumulation (Steinberg et al., 2006; Watt et al., 2006; Choi et al., 2007; Bruce et al., 2009), this is an extremely controversial area of active debate with numerous studies reporting conflicting findings. As a matter of fact, the original work of Philip Randle and colleagues in the 1960s demonstrated that an increase in fatty acid oxidation reduces glucose oxidation and subsequent glucose uptake in the isolated perfused heart and diaphragm, though these conclusions were simply extrapolated to skeletal muscle (Randle et al., 1963). In contrast, meticulous work from the Shulman laboratory suggests that fatty acid oxidation rates are impaired in muscle in the setting of obesity, and that accelerating fatty acid oxidation may improve glucose homeostasis in obesity via reducing the accumulation of lipid metabolites and subsequent inhibition of insulin signaling (Choi et al., 2007; Savage et al., 2007; Zhang et al., 2007). Similarly, muscle overexpression of CPT-1 via electroporation of an
adenovirus increases fatty acid oxidation rates and reduces membrane accumulation of DAG/ceramide, which results in a significant improvement in insulin signaling and glucose uptake in rats fed a HFD (Bruce et al., 2009). Indeed, chronic inhibition of CPT-1 in rats with etomoxir increases muscle lipid accumulation, which is associated with a worsening of obesity-induced insulin resistance (Dobbins et al., 2001). On the other hand, we have shown in a mouse model of diet-induced obesity that a 4-week treatment with the CPT-1 inhibitor, oxfenicine, reverses insulin resistance and glucose intolerance (Keung et al., 2013). Reasons for this discrepancy are not clear, but may be due to species’ related differences, or due to CPT-1 inhibition being provided at the onset of high fat feeding in the etomoxir study (Dobbins et al., 2001), whereas we allowed mice to become obese and insulin resistant before treating with oxfenicine (Keung et al., 2013). In support of our studies, Finck et al. have shown that elevated fatty acid oxidation rates in muscle-specific PPARα overexpressing mice induce insulin resistance, and that this can also be improved via treatment with the CPT-1 inhibitor, oxfenicine (Finck et al., 2005). Studies in humans consuming a HFD for 3 days have recapitulated these effects, as treatment with etomoxir (5 doses totaling 600 mg spread over 36 hrs) inhibited fatty acid oxidation, which was associated with a corresponding increase in glucose oxidation, sarcolemmal GLUT4 content in muscle fibers, and a lowering in HOMA index scores (Timmers et al., 2012). A key difference between our observations and the aforementioned studies where inhibiting fatty acid oxidation has beneficial effects on muscle insulin sensitivity involves the method of inhibition. Indeed, we used a 3-KAT inhibitor (trimetazidine) in this particular study, whereas the other studies inhibited fatty acid oxidation secondary to an inhibition of CPT-1 and subsequent mitochondrial fatty acid uptake (Finck et al., 2005; Timmers et al., 2012; Keung et al., 2013). It has been suggested that lipid overload
specifically in the mitochondria elicits mitochondrial dysfunction, increases oxidative stress, and impairs insulin sensitivity in muscle (Koves et al., 2008; Anderson et al., 2009). Thus, restricting mitochondrial fatty acid uptake versus directly inhibiting mitochondrial β-oxidation should not yield equivalent biological outcomes, as lipids may still enter the mitochondria if the inhibition takes place at the level of β-oxidation. Nevertheless, the discordant results of these multiple studies collectively illustrates the need for further work to elucidate exactly how alterations in fatty acid oxidation affect the development and progression of muscle insulin resistance.

The fact that intramuscular ceramide and DAG content did not increase further following 3-KAT inhibition may also explain why insulin resistance was not exacerbated in our model. At the same time, it may also be possible that an additional accumulation of muscle lipid metabolites (ie. 10-20% further increase) would not impart any further damage on glucose/insulin tolerance beyond that already attributed to the muscle lipid metabolites accumulated following HFD-induced obesity. Indeed, our model of HFD-induced obesity causes a doubling in the levels of muscle long chain acyl CoA compared to standard chow fed mice (Ussher et al., 2010), and hence the additional increase following trimetazidine treatment may not yield any further consequences on glucose tolerance and insulin sensitivity.

Though inhibition of 3-KAT-regulated fatty acid oxidation may not be harmful in our study, it is important to note that the inhibition of fatty acid oxidation in liver may have detrimental effects, potentially accelerating steatosis development (Savage et al., 2007). Nonetheless, we did not observe any further increase in hepatic TAG, long chain acyl CoA, and ceramide content in trimetazidine treated HFD-induced obese mice. Adiposity was also similar in our study, as treatment of obese mice with trimetazidine did not increase epididymal or
perirenal fat pad weight. Conversely, it is also possible that life-long modest reductions in fatty acid oxidation, such as which may occur in obese patients with ischemic heart disease who may be treated with trimetazidine over years or decades, may ultimately result in impaired insulin sensitivity. However, the answer to that question cannot be determined from this study, which only investigated the effects of a 3-week treatment regimen with trimetazidine on HFD-induced metabolic dysfunction. This treatment duration was chosen as we were concurrently investigating a separate cardiovascular study, determining whether trimetazidine therapy could alleviate obesity-induced cardiac dysfunction. Ultrasound echocardiography analyses at day 20 post-treatment revealed a significant amelioration of diet-induced cardiac hypertrophy (6.43 ± 0.50 g/mm vs. 4.98 ± 0.46 g/mm LV mass/tibia length) and contractile dysfunction in HFD-induced obese mice receiving trimetazidine (data not shown). These results are consistent with findings from Tuunanen et al. (Tuunanen et al., 2008), whereby treatment of idiopathic dilated cardiomyopathy patients with trimetazidine caused a modest 10% decrease in myocardial fatty acid oxidation rates, but significantly improved LV function. These authors also showed that trimetazidine inhibits endogenous TAG-derived fatty acid oxidation, consistent with the trend to increased gastrocnemius TAG content we observed in HFD-induced obese mice treated with trimetazidine (Bucci et al., 2011). Therefore, it appears that the antianginal agent trimetazidine elicits cardioprotection in the setting of obesity without adversely affecting muscle insulin sensitivity, though a more prolonged treatment may yield different results.

Overall, our results reveal important insights regarding the contribution of fatty acid oxidation to insulin resistance. It has been postulated that impaired skeletal muscle fatty acid oxidation can cause insulin resistance (Savage et al., 2007). Thus, treatment with trimetazidine in obese mice would be anticipated to exacerbate skeletal muscle insulin resistance, findings
which we did not observe, suggesting that trimetazidine would not impair insulin sensitivity in obese patients with angina. Furthermore, our observations in vitro demonstrate that unlike the heart, trimetazidine’s effect on muscle fatty acid oxidation is extremely mild. In spite of the ongoing debate concerning reduced fatty acid oxidation rates and insulin resistance, our findings do not raise caution regarding the use of trimetazidine to treat obese patients with angina.

ACKNOWLEDGEMENTS

We thank the dedicated staff of the Metabolomics and Biomarker Core of the Sarah W. Stedman Nutrition and Metabolism Center for measurement of acylcarnitines and tricarboxylic acid intermediates, and the Cardiovascular Translational Research Centre HPLC Core Facility for the measurement of long-chain acyl CoAs and ceramides.

AUTHOR CONTRIBUTIONS

Participated in research design: Ussher and G.D. Lopaschuk.


Performed data analysis: Ussher, Jaswal, Muoio, and G.D. Lopaschuk.

Wrote or contributed to the writing of the manuscript: Ussher, Keung, Jaswal, Muoio, and G.D. Lopaschuk.

DISCLOSURES

The authors have no conflicts to disclose.
REFERENCES


* This study was funded by a grant to GDL from the Heart and Stroke Foundation of Alberta and a grant to DMM from the National Institutes of Health (R01-HL101189). JRU is a trainee of the Alberta Heritage Foundation for Medical Research.
FIGURE LEGENDS

Figure 1: Schematic outlining the trimetazidine treatment protocol and physiological parameters tested in HFD-induced obese mice.

26 week-old C57BL/6NCrl mice were fed a high fat diet for 10 weeks. At the end of week 10, animals were administered trimetazidine hydrochloride (15 mg/kg/day) or saline by i.p. injection for 22 days. A glucose tolerance test (GTT) was performed at 7-days and 21-days post-treatment, while an insulin tolerance test (ITT) was performed at 14-days post-treatment. At 16/17-days post-treatment, mice underwent indirect calorimetry in metabolic cages, and were subjected to an exercise tolerance study at 11-days post-treatment. At study completion, ad libitum animals were sacrificed for tissue extraction and plasma collection.

Figure 2: Treatment with trimetazidine has no effect on body weight in HFD-induced obese mice, but induces a shift in energy substrate metabolism away from fatty acids and towards carbohydrates as an oxidative energy source in vivo.

A: Body weight and, B: Glucose intolerance in mice following 10 weeks of high fat feeding. C: Body weight of HFD-induced obese mice at 7-days post-treatment. D: Epididymal and E: Perirenal fat pad weight (normalized to body weight) at animal sacrifice in saline and trimetazidine treated HFD-induced obese mice. F: Plasma triacylglycerols (TAGs), and G: Free fatty acids (FFAs) in the ad libitum (day 22 post-treatment) and fasted state (day 21 post-treatment) from HFD-induced obese mice treated with saline or trimetazidine. H/I: Respiratory exchange ratio, J: Whole body oxygen consumption rates, K: Whole body heat production, and L: Locomotor activity in HFD-induced obese mice at day 16/17 post-treatment. Values
represent mean ± SE (n = 5 - 6). The significance of differences between 2-groups was
determined by the use of an unpaired, two-tailed Student’s $t$-test. The significance of
differences for multiple comparisons was estimated by two-way analysis of variance
(ANOVA). When ANOVA revealed differences, multiple t-tests with a Bonferroni correction
were performed on the data sets. *$P<0.05$, significantly different from lean/pre-HFD mice.
†$P<0.05$, significantly different from saline treated HFD-induced obese mice.

**Figure 3:** Treatment with trimetazidine does not affect glucose and insulin tolerance in HFD-
induced obese mice.

A: Day 7 and B: Day 21 glucose tolerance in trimetazidine treated HFD-induced obese mice. C:
Absolute change in blood glucose levels (mM) during an insulin tolerance test in trimetazidine
 treated HFD-induced obese mice 14-days post-treatment. D: % change in blood glucose levels
during the insulin tolerance test 14-days post-treatment. Values represent mean ± SE (n = 6).

**Figure 4:** Trimetazidine selectively inhibits fatty acid oxidation in vitro in a cell-specific
manner.

Oxidative metabolism (glucose and palmitate oxidation) in the A: Isolated working mouse
heart, B: Neonatal cardiomyocytes, and C: C2C12 myotubes treated with trimetazidine (100
µM in the isolated working heart and 10 µM in the cultured cells). Oleate oxidation rates in
primary cultures of human skeletal muscle myocytes treated with increasing concentrations of
either D: trimetazidine, or E: oxfenicine. Values represent mean ± SE (n = 3 - 6). The
significance of differences between 2-groups was determined by the use of an unpaired, two-
tailed Student’s $t$-test. The significance of differences for multiple comparisons was estimated
by two-way analysis of variance (ANOVA). When ANOVA revealed differences, multiple t-tests with a Bonferroni correction were performed on the data sets. *P<0.05, significantly different from saline treated counterpart.

**Figure 5:** Muscle lipid metabolite accumulation and levels of tricarboxylic acid cycle intermediates in HFD-induced obese mice following 22 days of treatment with trimetazidine.  
A: Gastrocnemius triacylglycerol content, B: Long-chain acyl CoA content, C: Diacylglycerol content, D: Ceramide content, E: Tricarboxylic acid (TCA) cycle intermediate levels, F: Medium chain acylcarnitine content, and G: Long-chain acylcarnitine content in saline and trimetazidine treated HFD-induced obese mice. H/I: Plasma long-chain acylcarnitine content in saline and trimetazidine treated HFD-induced insulin resistant mice. Values represent mean ± SE (n = 6). Differences were determined by the use of an unpaired, two-tailed Student’s t-test. *P<0.05, significantly different from HFD-induced insulin resistant saline treated mice.

**Figure 6:** Hepatic lipid metabolite accumulation in HFD-induced obese mice following 22 days of treatment with trimetazidine.  
A: Hepatic triacylglycerol content, B: Long-chain acyl CoA content, and C: Ceramide content in saline and trimetazidine treated HFD-induced insulin resistant mice. Values represent mean ± SE (n = 6).
Table 1. Plasma parameters following trimetazidine treatment of HFD-induced obese mice

<table>
<thead>
<tr>
<th></th>
<th>Ad libitum saline</th>
<th>6 hr fast saline</th>
<th>Ad libitum trimetazidine</th>
<th>6 hr fast trimetazidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td>7.95 ± 0.70</td>
<td>7.13 ± 0.29</td>
<td>7.83 ± 0.43</td>
<td>7.52 ± 0.56</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>4.41 ± 0.62</td>
<td>0.92 ± 0.11</td>
<td>4.21 ± 0.34</td>
<td>0.95 ± 0.08</td>
</tr>
<tr>
<td>TAG (mg/dL)</td>
<td>240.65 ± 12.52</td>
<td>254.89 ± 11.65</td>
<td>298.05 ± 16.24*</td>
<td>258.33 ± 12.26</td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.38 ± 0.04</td>
<td>0.47 ± 0.04</td>
<td>0.33 ± 0.06</td>
<td>0.43 ± 0.03</td>
</tr>
</tbody>
</table>

Plasma parameters in trimetazidine treated HFD-induced obese mice. Ad libitum plasma was collected on day 22 post-treatment 2 hrs into the dark cycle during animal euthanization, whereas fasted plasma was collected following a 6 hr fast on day 21 post-treatment (n = 5-6). Values represent means ± S.E.M. *P<0.05, indicates a significant difference from saline treated counterpart. FFA = free fatty acid, TAG = triacylglycerol.
Figure 1

22 days HFD + Trimetazidine (15 mg/kg IP, once daily) or Saline

10 wks HFD (60% kcal from lard)

- GTT
- Exercise Tolerance
- ITT
- Metabolic Cages
  - RER
  - VO2
  - Heat Production
  - Locomotor Activity

GTT
Animal Sacrifice
- Plasma Collection
- Tissue Extraction
- Metabolomics
- Lipid Metabolite Extraction
**Figure 2**

**A**

Body Weight (g)

- Pre-HFD
- 10-wk HFD

**B**

Pre-Treatment Glucose Tolerance After 10-wks HFD

- Saline
- Trimetazidine
- Lean

**C**

% of Initial Body Wt

- Post 1-wk Treatment

**D**

Epididymal Fat

- % of Body Weight

**E**

Perirenal Fat

- % of Body Weight

**F**

Plasma TAGs (mg/dl)

- Fed
- Fasted

**G**

Plasma FFAs (mM)

- Fed
- Fasted

**H**

Light Cycle

- RER

- P < 0.01

**I**

Injection

- RER

- Dark Cycle
- Light Cycle

**J**

O2 Consumption (ml/kg/hr)

- Injection

- Dark Cycle
- Light Cycle

**K**

Injection

- Heat Production (kcal/hr)

- Dark Cycle
- Light Cycle

**L**

24 hr Locomotor Activity (beam breaks)

- 0 to 15,000

---

*Fat Pad Weight at Animal Sacrifice*

*6 Hr Fasted Plasma at Day 21 & Ad Libitum Plasma at Day 22*

*Post-Treatment*

*Indirect Calorimetry Assessment at Day 17 Post-Treatment*
Figure 3

A. Glucose Tolerance

B. Glucose Tolerance

C. Insulin Tolerance

D. Insulin Tolerance
Figure 4

A. 60 Min Aerobic Perfusion
- (5.0 mM glucose/0.4 mM palmitate)
- [9,10^-3H]palmitate/[U-14C]glucose

- 100μM trimetazidine present throughout entire 60 min perfusion

B. 24 Hr Treatment
- (11.0 mM glucose/0.8 mM palmitate)
- [1-14C]palmitate or [U-14C]glucose added to media for final 3 hrs of treatment

- 10μM trimetazidine present during final 3 hrs of treatment

C. 24 Hr Treatment
- (11.0 mM glucose/0.8 mM palmitate)
- [1-14C]palmitate or [U-14C]glucose added to media for final 3 hrs of treatment

- 10μM trimetazidine present during final 3 hrs of treatment

D. 1 Hr Pretreatment
- 3 Hr Treatment
- (5.5 mM glucose/0.3 mM oleate)
- [1-14C]oleate

- Trimetazidine or oxfenicine treatment 1 hr prior to assessment of fatty acid oxidation and present throughout duration of assay

E. 1 Hr Pretreatment
- 3 Hr Treatment
- (5.5 mM glucose/0.3 mM oleate)
- [1-14C]oleate

- Trimetazidine or oxfenicine treatment 1 hr prior to assessment of fatty acid oxidation and present throughout duration of assay

F. Trimetazidine Treated Human Myotubes (n = 3)
- 0, 1.3nM, 6nM, 32nM, 0.16μM, 0.8μM, 4μM, 20μM

- 0, 1.3nM, 6nM, 32nM, 0.16μM, 0.8μM, 4μM, 20μM

- nmol FA/mg/hr

G. Oxfenicine Treated Human Myotubes (n = 3)
- 0, 512nM, 2.6μM, 13μM, 64μM, 320μM, 1.6mM, 8mM

- 0, 512nM, 2.6μM, 13μM, 64μM, 320μM, 1.6mM, 8mM

- nmol FA/mg/hr

- Saline □ Trimetazidine ■
Figure 5

A. Triacylglycerol (n = 6)  
B. Long Chain Acyl CoA (n = 6)  
C. Diacylglycerol (n = 6)  
D. Ceramide (n = 6)  

E. TCA Cycle Intermediates (n = 6)  
F. Gastrocnemius Medium Chain Acylcarnitines (n = 6)  

G. Gastrocnemius Long Chain Acylcarnitines (n = 6)  

H. Plasma Long Chain Acylcarnitines (n = 6)  
I. Total Plasma Long Chain Acylcarnitines (n = 6)  

---

Plasma & Muscle Lipid Profiling Following 22 Days of Treatment with Trimetazidine
Figure 6

A. Hepatic Triacylglycerol (n = 6)

B. Hepatic Long Chain Acyl CoA (n = 6)

C. Hepatic Ceramide (n = 6)

Hepatic Lipid Profiling Following 22 Days of Treatment with Trimetazidine