Insulin-stimulated cardiac glucose oxidation is increased in high fat diet-induced obese mice lacking malonyl CoA decarboxylase

Running Title: Myocardial triglycerides and glucose oxidation

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Objective: While an impaired ability to oxidize fatty acids is thought to contribute to intracellular lipid accumulation, insulin-resistance, and cardiac dysfunction, high rates of fatty acid oxidation could also impair glucose metabolism and function. We therefore determined the effects of diet-induced obesity (DIO) in wild type (WT) mice and mice deficient for malonyl CoA decarboxylase (MCD-/-) (an enzyme promoting mitochondrial fatty acid oxidation), on insulin-sensitive cardiac glucose oxidation.

Research Design and Methods: WT and MCD-/- mice were fed a low fat or high fat diet for 12 weeks and intra-myocardial lipid metabolite accumulation was assessed. A parallel feeding study was performed to assess myocardial function and energy metabolism (nmol/g dry wt/min) in isolated working hearts (+/- insulin).

Results: DIO markedly reduced insulin-stimulated glucose oxidation compared to low fat fed WT mice (167 ± 31 vs. 734 ± 125, \( P < 0.05 \)). MCD-/- mice subjected to DIO displayed a more robust insulin-stimulated glucose oxidation (554 ± 82 vs. 167 ± 31, \( P < 0.05 \)), and displayed less incomplete fatty acid oxidation, evidenced by a decrease in long chain acylcarnitines compared to WT counterparts. MCD-/- had similar long chain acyl CoAs as WT mice subjected to DIO, but had increased triacylglycerol (TG) levels (10.92 ± 3.72 vs. 3.29 ± 0.62 µmol/g wet wt, \( P < 0.05 \)).

Conclusions: DIO does not impair cardiac fatty acid oxidation or function, and we demonstrate disassociation between myocardial lipid accumulation and insulin sensitivity. Our results suggest that MCD deficiency is not detrimental to the heart in obesity.
The incidence of obesity, insulin resistance, and diabetes is rapidly increasing (1-4), and by 2025 it is estimated that worldwide the incidence of diabetes will affect nearly 333 million individuals between 20 and 79 years of age (41). Obesity, insulin resistance, and diabetes are characterized, at least in part, by an elevation of circulating plasma fatty acid concentrations. While there is a general consensus that increased circulating fatty acids lead to increased rates of fatty acid uptake in muscle, the contribution of impaired mitochondrial fatty acid oxidation to the accumulation of cytosolic intramuscular fatty acid metabolites implicated in the pathogenesis of insulin resistance (such as long chain acyl CoAs, triacylglycerols (TG), ceramides, and diacylglycerols) remains controversial (5-8). It has been proposed that an acceleration of mitochondrial fatty acid oxidation may alleviate or attenuate skeletal muscle insulin resistance by preventing the cytosolic accumulation of these fatty acid metabolites (9; 10).

We have recently demonstrated that insulin resistance in response to high fat feeding (diet-induced obesity (DIO)) in skeletal muscle does not arise from an impairment of mitochondrial fatty acid oxidation per se, but rather is associated with excessive rates of incomplete fatty acid oxidation (11; 12). DIO leads to an accumulation of intramuscular long chain acyl CoAs, the vast majority of which are localized and oxidized in the mitochondria (13). However, in the absence of high-energy demand, often observed in obese, sedentary individuals, flux through the (tricarboxylic acid) TCA cycle is unable to increase to such a rate as to accommodate the large increase in acyl CoAs oxidized. Interestingly, we also demonstrated in mice that preventing the mitochondrial uptake of fatty acids attenuates the development of fatty acid-induced whole body insulin resistance (11). This was achieved by genetic deletion of malonyl CoA decarboxylase (MCD-/-), which degrades malonyl CoA, a potent endogenous inhibitor of the rate limiting enzyme in mitochondrial fatty acid uptake (carnitine palmitoyl transferase-I). A similar beneficial effect of MCD inhibition on insulin stimulated glucose metabolism in skeletal muscle cells was also recently demonstrated (14). In our studies, MCD inhibition was associated with a reduction in the accumulation of long chain acylcarnitines, and a prevention in the downregulation of TCA cycle intermediates observed during DIO (11). It can be argued that such a strategy would increase the intramuscular accumulation of the aforementioned fatty acid metabolites (15). However, there was not any increase in long chain acyl CoAs and TG beyond that induced by high fat feeding itself.

The accumulation of intra-myocardial fatty acid metabolites is also implicated in mediating myocardial dysfunction (16; 17). It is not clear what effect modifying fatty acid oxidation has on insulin sensitivity in cardiac muscle. Previous findings in the obese Zucker rat demonstrate an impairment in fasting myocardial fatty acid oxidation compared to lean controls, an effect accompanied by elevated levels of intra-myocardial lipid and an inability to increase the expression of peroxisome proliferator activated receptor alpha (PPARα) target genes (17). Conversely, we previously showed no difference in the rates of myocardial fatty acid oxidation in either the fed or fasted states between insulin resistant JCR:LA-cp rats and lean controls (18). Furthermore, in insulin resistant JCR:LA-cp rats there was nearly a doubling in myocardial TG stores, supporting the observation that the accumulation of intra-myocardial fatty acid metabolites is the result of excessive fatty acid supply, as opposed to impaired fatty acid oxidation.
oxidation. As well, we have demonstrated that myocardial fatty acid oxidation rates are elevated in transgenic PPARα overexpressing mice, a phenotype resembling that of type 2 diabetes (19). Therefore, debate still exists with regards to the accumulation of fatty acid metabolites, fatty acid oxidation rates, and myocardial insulin resistance.

In this study we investigated the relationship between myocardial fatty acid metabolite accumulation, fatty acid oxidation, and insulin-stimulated glucose oxidation in wild-type (WT) and MCD-/- mice fed either low or high fat diet (DIO). We hypothesized that inhibition of mitochondrial fatty acid uptake via ablation of MCD preserves insulin-sensitive myocardial glucose oxidation, despite elevated levels of intra-myocardial long chain acyl CoAs and TG.

RESEARCH DESIGN AND METHODS:

An expanded Research Design and Methods section is available in the online data supplement at http://diabetes.diabetesjournals.org. Details on isolated working heart perfusions, ultrasound echocardiography, metabolic profiling, high energy phosphate assessment, PDH activity, and immunoblot analysis is also provided in the online data supplement.

Animal Studies: All animals received care according to the Canadian Council on Animal Care and the University of Alberta Health Sciences Animal Welfare Committee. Twelve week old WT or MCD-/- mice were placed on a standard chow/low fat diet (4% kcal from lard) or high fat diet (60% kcal from lard, Research Diets; D12492) for a 12 week period. At the end of week 12, animals were euthanized via an intraperitoneal injection of sodium pentobarbital (12 mg) either in the fed state in the middle of the dark cycle, or after a 6 hr fast. Hearts were excised and immediately frozen in liquid N₂ for biochemical analyses, or perfused in the isolated working mode (see expanded


RESULTS:

Effects of DIO on cardiac fatty acid and glucose oxidation in isolated working mouse hearts: Subjecting mice to a 10-week period of DIO did not result in any alteration in isolated working heart function compared to low fat fed mice (Table 1). Of interest is that cardiac function was also normal in MCD-/- mice regardless of whether they were obtained from low fat fed or DIO group. This demonstrates that MCD deficiency does not contribute to cardiac dysfunction in DIO mice. Reinforcing our ex vivo perfusion data, we also showed via ultrasound echocardiography that in vivo cardiac function was unaltered by DIO in WT or MCD-/- mice (Table 2). Despite the lack of functional changes, dramatic differences in cardiac energy metabolism were observed between the experimental groups. Hearts from WT mice subjected to DIO had a significant reduction in glucose oxidation rates compared to low fat fed mice, with no change in oxidation of exogenously supplied [9,10-³H]palmitate (Figure 1B). Insulin (100 µU/mL) robustly stimulated myocardial glucose oxidation in low fat fed WT mice (Figure 1A), and decreased fatty acid oxidation (Figure 1B). In contrast, insulin had only a small effect on glucose oxidation in WT DIO mice (Table 3), demonstrating an impaired insulin-stimulated glucose metabolism in these DIO hearts.

Insulin stimulation of glucose oxidation was significantly increased in MCD-/- mice compared to WT mice, in both the low fat fed and DIO groups (Figure 1A). This was accompanied by a significant decrease in fatty acid oxidation in low fat fed MCD-/- mice, and a small non-significant decrease in fatty acid oxidation in MCD-/- DIO mice, which may be attributed to a trend
towards lower fatty acid oxidation rates in the absence of insulin (Figure 1B). As such, the contribution of glucose oxidation for acetyl CoA production was significantly increased, whereas the contribution of fatty acid oxidation for acetyl CoA production was significantly decreased in MCD-/− DIO mice, illustrating that these mice rely less on fatty acids for energy metabolism versus their WT counterparts (Figure 1C). Paralleling our earlier work in MCD-/− mice whereby compensatory increases in PPARα target gene mRNA expression could explain the lack of change in myocardial fatty acid oxidation rates in MCD-/− mice, we show that MCD-/− DIO hearts had higher PPARα target gene, PDK4, protein expression (Figure 2A). Despite this elevation in PDK4 expression, total PDH activity and the active state of PDH were significantly higher in hearts from MCD-/− DIO hearts, but the ratio of active to total PDH activity remained the same in both groups (Figure 2B-2D). Nonetheless, these findings demonstrates that decreasing MCD can partially overcome the dramatic impairment in insulin-stimulated glucose metabolism seen with DIO, which may be due to a preservation of PDH activity.

Effects of DIO and fasting on cardiac malonyl CoA levels: Since malonyl CoA is a major regulator of cardiac fatty acid oxidation, we examined what effect DIO had on cardiac malonyl CoA levels. As shown in Figure 3, DIO had no effect on malonyl CoA levels in WT mice compared to low fat fed WT mice. As expected, MCD-/− subjected to DIO showed a significant increase in malonyl CoA levels compared to WT DIO mice (Figure 3). In addition, malonyl CoA levels from fasted WT mice did not differ from their fed counterparts, but were significantly lower than those in fasted MCD-/− mice (Supplementary Figure 1A).

Effects of DIO and fasting on myocardial long chain acylcarnitine levels: Following 12 weeks of low fat feeding or DIO, there was a significant reduction in the accumulation of a number of long chain acylcarnitine species in hearts from MCD-/− mice compared to hearts from WT littermates (Figure 4A-4D). In addition, medium chain fatty acids do not require CPT1 for entry into the mitochondria, and as such, no changes in any medium chain acylcarnitine species were observed in hearts of MCD-/− DIO mice versus WT DIO mice, except the C10:3 species (4.4 ± 1.3 vs. 15.8 ± 4.1 pmol C10:3 acylcarnitine/mg protein, P<0.05, data not shown). These findings are consistent with an inhibition of CPT1, and a lowering of intramitochondrial fatty acid oxidation intermediates.

In another group, following 12 weeks of low fat feeding, mice were fasted for 6 hours prior to the analysis of myocardial long chain acylcarnitines, and similar to our DIO findings, we report that long chain acylcarnitines do not accumulate to the same extent in hearts from fasted MCD-/− mice versus their WT counterparts (Supplementary Figure 1B). The medium chain acylcarnitine profile from fasted mice also paralleled our DIO findings, where no accumulation was observed except for the C10:3 species (4.8 ± 2.2 vs. 17.9 ± 2.0 pmol C10:3 acylcarnitine/mg protein, P<0.05, data not shown), consistent with no CPT1 requirement for mitochondrial oxidation. Interestingly, the fasting induced increase in β-hydroxybutyrl (C4-OH) carnitine was prevented in MCD-/− group (223.9 ± 20.8 vs. 104.2 ± 12.7 pmol β-hydroxybutyrl carnitine/mg protein, P<0.05, data not shown), paralleling what we have previously reported in the skeletal muscle (11).

Myocardial TCA cycle intermediates are not depleted by DIO or fasting: Previous work in skeletal muscle has suggested that fatty acid overload in response to fasting or DIO results in incomplete fatty acid oxidation by causing a disconnect between fatty acid oxidation and the TCA cycle, an effect
prevented by the genetic deletion of MCD (11). Interestingly, myocardial short chain CoAs and TCA cycle intermediates were not depleted by either fasting (data not shown) or DIO (Figure 4E and 4F) in WT or MCD-/- groups, contrasting what we have previously reported in skeletal muscle. This likely reflects the higher acetyl CoA production from glucose oxidation in the MCD-/- mice, however, total acetyl CoA production from both glucose and palmitate was significantly depressed in WT DIO mice, indicative of mitochondrial impairment (Table 4). Moreover, deficiency of MCD, although associated with a reduced contribution of fatty acids for acetyl CoA production, did not impair mitochondrial function in lean mice, and did not exacerbate the impairment observed in DIO WT mice. AMP (0.37 ± 0.08 vs. 0.41 ± 0.06 µmol/g dry weight in WT and MCD-/- low fat fed, and 0.31 ± 0.05 vs. 0.33 ± 0.03 µmol/g dry weight in WT and MCD-/- DIO) and ATP (1.89 ± 0.15 vs. 1.81 ± 0.35 µmol/g dry weight in WT and MCD-/- low fat fed, and 1.63 ± 0.11 vs. 1.77 ± 0.27 µmol/g dry weight in WT and MCD-/- DIO) levels were also not altered between any of the experimental groups, consistent with normal TCA cycle activity in these hearts. Myocardial lactate and pyruvate levels were also measured and showed no changes, regardless of diet, between WT and MCD-/- mice (Table 5).

**Intra-myocardial fatty acyl CoA and TG profile following DIO or fasting:** It has been suggested that an impairment in the mitochondrial uptake and subsequent oxidation of fatty acids contributes to the accumulation of intramuscular fatty acyl CoAs and the development of insulin resistance (6; 8; 15) and myocardial dysfunction (16; 17; 20). Therefore, we investigated the effects of DIO on the myocardial accumulation of fatty acyl CoAs. In low fat fed mice, MCD deficiency did not alter the levels of the major long chain acyl CoAs or total long chain acyl CoAs compared to WT mice (Figure 5A). DIO resulted in a marked increase in the major cardiac long chain acyl CoAs and total long chain acyl CoAs in WT mice compared to low fat fed mice (Figure 4A). DIO significantly increased intra-myocardial long chain acyl CoA levels to similar extents in MCD-/- mice compared to WT mice (Figure 5A). This demonstrates dissociation between long chain acyl CoA accumulation and insulin-sensitivity in DIO mice.

MCD deficiency did not alter myocardial TG levels in low fat fed mice (Figure 5B). However, a significant increase in TG was seen in the DIO MCD-/- mice compared to WT DIO mice. This again demonstrates a disassociation between accumulation of TG and insulin resistance in DIO hearts.

Since accumulation of ceramide has also been implicated in cardiac lipotoxicity, we measured ceramide levels in WT and MCD-/- subjected to DIO (Figure 5C). No difference was observed in ceramide levels between DIO and low fat fed mice in either WT or MCD-/- groups.

Low fat fasted WT and MCD-/- mice showed similar results to DIO WT and MCD-/- mice, where a similar accumulation in long chain acyl CoA and no accumulation in ceramide was observed. Furthermore, myocardial TG once again did not accumulate in fasted WT mice, but did accumulate in fasted MCD-/- mice (8.42 ± 1.28 vs. 3.72 ± 0.68 µmol/g wet wt, P<0.05, data not shown).

**Effects of DIO on expression and phosphorylation of proteins involved in regulating myocardial energy metabolism and insulin signaling:** Several proteins including peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) and uncoupling protein 2 (UCP2) have been proposed to be altered by DIO in both skeletal and cardiac muscle (12; 21-24). Neither the expression of PGC1α (Figure 6A) or UCP2
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(Figure 6B), was altered by DIO in either the WT and MCD-/- groups. Supporting improved cardiac insulin sensitivity in MCD-/- DIO mice, we demonstrate an increase in Akt serine 473 phosphorylation and a trend to an increased GSK3β serine 9 phosphorylation in MCD-/- DIO hearts (P=0.09) perfused aerobically with insulin (Figure 7A and 7B). Insulin had no effect on 5’AMP activated protein kinase (AMPK) phosphorylation at threonine 172 (Figure 7C), suggesting that AMPK likely does not play a role in the improved insulin sensitivity observed in MCD-/- DIO hearts.

DISCUSSION:

This study provides a number of important novel findings regarding the effects of DIO on cardiac insulin sensitivity. We demonstrate that: 1) DIO results in a profound impairment in insulin-stimulated glucose metabolism in mouse hearts, 2) increasing malonyl CoA levels in the heart secondary to deletion of MCD can prevent this impairment, 3) the DIO-induced impairment in cardiac insulin-stimulated glucose metabolism can be dissociated from the accumulation of myocardial lipid intermediates, contradicting what has been previously proposed in skeletal muscle, 4) similar to recent evidence in skeletal muscle (11), this impairment in insulin-stimulated glucose metabolism correlates with the accumulation of intermediates of incomplete fatty acid oxidation, and 5) the insulin sensitizing effects of MCD deletion are accompanied by a decrease in the accumulation of fatty acid intermediates of incomplete fatty acid oxidation. These data provide important insights into the pathophysiology of cardiac insulin resistance, and suggest that inhibition of fatty acid oxidation (as opposed to stimulation of fatty acid oxidation) may have therapeutic potential in preventing obesity-induced impairments on cardiac insulin-stimulated glucose metabolism.

In skeletal muscle, two opposing views of how high fat induced insulin resistance occurs have been proposed. A widely cited hypothesis is that the accumulation of cytoplasmic fatty acid intermediates impairs insulin signaling (6; 8; 15). Based on this hypothesis, it has been proposed that stimulation of fatty acid oxidation can lower these intermediates and improve insulin sensitivity. However, recent studies by us (11) and others (14) have challenged this concept. Recent studies have suggested that accumulation of intermediates of incomplete fatty acid oxidation correlate with skeletal muscle insulin-resistance (11; 25; 26). Of importance is that inhibition of fatty acid oxidation secondary to MCD deletion resulted in an improvement of whole body insulin-sensitivity as determined via glucose/insulin tolerance testing, fasting plasma glucose levels, and indirect calorimetry (11). Using siRNA to knockdown MCD in skeletal muscle cells also results in an increase in insulin stimulated glucose metabolism (14). In this study we show a similar beneficial effect of MCD deletion in cardiac muscle. MCD-/- mice subjected to DIO showed a significant improvement in insulin stimulated cardiac glucose oxidation and insulin signaling at the level of Akt phosphorylation. This was associated with a decreased accumulation in a number of long chain acyl carnitine species in response to DIO. These data clearly suggest that stimulation of cardiac fatty acid oxidation would not be desirable in preventing the impairment in insulin-stimulated glucose metabolism seen with DIO.

The preservation of insulin-stimulated glucose oxidation in hearts from MCD-/- mice may be due to increases in both active and total PDH activity versus that observed in the WT littermate controls. Interestingly, the percentage of active PDH versus total PDH activity remained the same in both MCD-/- and WT DIO hearts. However, the fact that
both the active portion and total activity of PDH are higher in the MCD-/- DIO hearts likely explains the preservation of insulin-stimulated glucose oxidation rates in these mice.

Surprisingly, we only observed a trend to a reduction in fatty acid oxidation rates in hearts from MCD-/- mice, regardless of diet. Nevertheless, it is important to emphasize that the fatty acid oxidation assay utilized in this study only measures oxidation of a single exogenously supplied fatty acid ([9,10-\textsuperscript{3}H]palmitate). Unfortunately, the assay does not account for incomplete fat oxidation, oxidation of endogenously supplied fatty acids and oxidation of other fatty acid species (oleate, stearate, etc). By comparison, the intra-myocardial acylcarnitine measurements provide more comprehensive information. The acylcarnitine profile revealed that MCD deficiency had a greater impact on the accumulation of oleyl-carnitine (C18:1), stearoyl-carnitine (C18:0), linoleoyl-carnitine (C18:2) and tetradecadienoyl-carnitine (C14:2) as compared to palmitoyl-carnitine (C16:0). Coupled together with the observation that ATP and acetyl CoA levels were similar between non-perfused WT and MCD-/- DIO hearts, despite greater glucose oxidation rates in MCD-/- DIO hearts, it is highly suggestive that fatty acid oxidation is greater in the hearts of WT mice.

In this study, DIO increased long chain acyl CoAs, while not affecting TG or ceramide levels in hearts from WT mice. MCD deletion did not exacerbate the increase in long chain acyl CoA’s in DIO mice, but did result in an increase in TG levels compared to WT DIO mice. DIO increased TG in hearts from MCD-/- mice. Whereas DIO severely attenuated insulin-stimulated glucose oxidation in hearts from WT mice, hearts from MCD-/- mice were protected from this decrement. These data demonstrate a clear dissociation between myocardial lipid accumulation and insulin resistance in DIO.

The DIO-induced alterations in myocardial fatty acid metabolite accumulation and insulin-stimulated glucose oxidation also occurred independently of changes in the expression of PGC1\(\alpha\) or UCP2, or the expression and phosphorylation of AMPK. These data indicate that inhibition of the mitochondrial uptake of fatty acids via ablation of MCD does not exacerbate the accumulation of intra-myocardial fatty acyl carnitines or fatty acyl CoAs, and does not induce insulin-resistance or myocardial dysfunction. Rather, ablation of MCD prevents the accumulation of intra-myocardial acylcarnitines likely by increasing the esterification of available intracellular fatty acids into TG, thereby alleviating the detrimental effects of DIO on cardiac insulin-stimulated glucose metabolism.

Supporting our findings, studies in mice with a cardiac specific overexpression of PPAR\(\alpha\) demonstrate an elevation in cardiac fatty acid oxidation rates and possess a phenotype mimicking that seen in type 2 diabetes (19). These animals have a dramatic impairment in insulin-stimulated glucose oxidation and show a reduction in \textsuperscript{18}F-fluorodeoxyglucose uptake via positron emission topography imaging. Furthermore, genetic ablation of the fatty acid transporter, CD36, in these animals restored the impairment in glucose oxidation rates, with a strong trend to a reduction in oxidation of exogenously supplied palmitate (27). Elegant studies by Aasum and colleagues also parallel our findings, where DIO mice and leptin receptor deficient (\textit{db/db}) diabetic mice have a dramatic impairment in insulin-stimulated glucose oxidation rates (28; 29). Peripheral activation of PPAR\(\alpha\) in DIO mice with fenofibrate improved whole body glucose tolerance and myocardial recovery from ischemia/reperfusion injury, while significantly increasing hepatic fatty acid oxidation rates (28). However, myocardial fatty acid oxidation rates were significantly
reduced, and this was associated with a complete restoration of glucose oxidation rates. Treatment of db/db mice with the PPARγ agonist, rosiglitazone, produced a similar effect, reducing myocardial fatty acid oxidation rates and restoring glucose oxidation rates to that seen in db/+ lean non-diabetic control mice, which was also associated with protection against myocardial ischemia/reperfusion injury (29).

Interestingly, in contrast to skeletal muscle (11), there was not an accompanying depletion of TCA cycle intermediates in hearts from WT mice subjected to DIO compared to low fat fed WT mice. We also did not observe any differences in total high-energy phosphates between groups. This discrepancy may be related to marked increase in acetyl CoA derived from glucose oxidation in the MCD/-/- mice hearts, which may exceed that seen in skeletal muscle (Table 3). Furthermore, potential differences in the anaplerotic capacity of cardiac and skeletal muscle may also partially account for this difference. Pyruvate carboxylation is an important anaplerotic reaction in cardiac muscle (30), and the relative abundance of pyruvate carboxylase is greater in cardiac versus skeletal muscle (31). Taken together these differences may, by mass action, replenish the TCA cycle at the level of malate and oxaloacetate (32-34) and thus account for the lack of TCA cycle intermediate depletion in response to fasting or DIO in hearts from both WT and MCD/-/- mice.

As discussed, it has been hypothesized that an acceleration of skeletal muscle fatty acid β-oxidation has the potential to ameliorate insulin resistance by preventing the cytosolic accumulation of fatty acid metabolites (9; 10). These findings are of particular importance as this hypothesis has been extrapolated to cardiac muscle, where it is proposed to attenuate myocardial dysfunction in the failing heart (16; 17; 20). Thus, it was initially anticipated that MCD/-/- mice would accumulate intramuscular lipid and become insulin resistant, moreover, that this would be exacerbated by DIO. However, this was clearly not the case, as MCD/-/- mouse hearts were highly insulin sensitive and protected against insulin resistance in response to DIO. We also show that although MCD/-/- mice accumulate intra-myocardial long chain acyl CoAs in response to DIO, this accumulation is not exacerbated compared to that seen in WT mice. The intra-myocardial content of ceramides was not affected by either fasting or DIO in hearts obtained from WT or MCD/-/- mice. The lack of alteration in myocardial ceramide(s) content may be related to the dynamic nature of this sphingolipid pool, the size of which is determined by both its rates of synthesis and degradation (35). The aforementioned effects are likely attributable to the partitioning of intracellular fatty acid intermediates into the TG pool in hearts from MCD/-/- mice following both fasting and DIO.

Despite the increased myocardial TG in hearts from MCD/-/- mice following DIO, myocardial insulin sensitivity was preserved, evidenced from the insulin-stimulated increase in myocardial glucose oxidation compared to hearts from WT mice following DIO. These findings support recent work in skeletal muscle suggesting that TG accumulation may not be a mediator of insulin resistance, but may actually serve as a buffer against other potential lipid mediators of insulin resistance such as ceramides and DAGs (36; 37). Further support for this concept lies in the “Athletes Paradox”, where endurance-trained individuals have greater muscle insulin sensitivity despite greater levels of intramuscular TG (38; 39). Although no changes in ceramide were observed in hearts from MCD/-/- mice following DIO, it may be possible that the increase in myocardial TG buffered against the build up of DAG in MCD/-/- mice. A recent study also demonstrates that acute inhibition of MCD in
human skeletal muscle cells via an siRNA approach decreases fatty acid uptake and oxidation, effects that are accompanied by a concomitant increase in glucose uptake and oxidation (14). Previous work from our laboratory demonstrates that the acute inhibition of MCD with novel MCD inhibitors and the genetic deletion of MCD also causes a metabolic shift in myocardial substrate preference towards increased glucose oxidation and improves the recovery of myocardial function following ischemia-reperfusion (40; 41).

The results of this study are in direct contrast to a recent study published by Essop and colleagues showing that insulin sensitivity was higher in mice deficient for acetyl CoA carboxylase 2 (ACC2-/-) (42). ACC2 produces malonyl CoA from acetyl CoA, hence ACC2-/- mice have lower malonyl CoA levels, and based on the Randle Cycle, should have decreased glucose oxidation rates at the expense of elevated fatty acid oxidation rates. Interestingly, ACC2-/- mice have elevated glucose and fatty acid oxidation rates, and an increased insulin-stimulated 2-deoxyglucose uptake. However, it should be noted in this study that the reported glucose oxidation rates are 10-20 fold lower than what is normally reported in the literature, and the change in fatty acid oxidation rates is very minimal, showing a smaller difference versus the fatty acid oxidation rates between WT and MCD-/- mice reported in our study.

In regards to AMPK, it has been shown to undergo alterations in both the muscle and hypothalamus following DIO, and may account for some of the metabolic changes that take place in response to DIO, due to an impaired response to leptin (43). Despite these findings, our results show no difference between AMPK phosphorylation in WT and MCD-/- DIO hearts, plus we have also previously shown that leptin does not activate AMPK in isolated perfused working rat hearts (44), suggesting that leptin is likely not mediating an effect on AMPK to alter glucose metabolism in this study.

In conclusion, these results demonstrate that DIO results in an impaired cardiac insulin sensitivity, which is not correlated with the accumulation of cytoplasmic lipid intermediates. MCD deficiency prevents this impairment, independent of lipid accumulation. The beneficial effects of MCD deficiency on improving insulin sensitivity correlate with a decrease in incomplete fatty acid oxidation. Thus, inhibition of mitochondrial fatty acid uptake via MCD inhibition represents a novel mechanism for treating many of the detrimental conditions associated with DIO.

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REFERENCES:
Table 1: Malonyl CoA decarboxylase (MCD) deficiency does not result in ex vivo cardiac dysfunction following DIO

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>WT (LF)</th>
<th>MCD-/- (LF)</th>
<th>WT (DIO)</th>
<th>MCD-/- (DIO)</th>
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<tr>
<td>Cardiac Output</td>
<td>8.3 ± 1.0</td>
<td>11.3 ± 1.1</td>
<td>11.2 ± 0.9</td>
<td>10.8 ± 0.3</td>
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<td>(mL/min)</td>
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<td>Cardiac Work</td>
<td>5.3 ± 0.8</td>
<td>7.4 ± 0.9</td>
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<tr>
<td>(mL/mm Hg/min)</td>
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Parameters of cardiac function (cardiac output and cardiac work) were assessed in isolated working hearts obtained from WT and MCD-/- mice subjected to either a low fat diet or DIO (n = 5-7). Values represent means ± SE. LF = Low fat, DIO = Diet induced obesity, MCD = malonyl CoA decarboxylase

Table 2: MCD deficiency does not result in in vivo cardiac dysfunction following DIO.

<table>
<thead>
<tr>
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<th>MCD-/- (LF)</th>
<th>WT (DIO)</th>
<th>MCD-/- (DIO)</th>
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<tr>
<td>Tei Index</td>
<td>0.42 ± 0.02</td>
<td>0.44 ± 0.02</td>
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<td>Cardiac Output</td>
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<td>26.19 ± 2.10</td>
<td>23.07 ± 0.71</td>
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<td>(mL/min)</td>
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<td>LVPW:d (mm)</td>
<td>0.74 ± 0.02</td>
<td>0.83 ± 0.06</td>
<td>0.87 ± 0.02*</td>
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<td>LVID:d (mm)</td>
<td>4.10 ± 0.10</td>
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<td>LVPW:s (mm)</td>
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<td>LVID:s (mm)</td>
<td>2.78 ± 0.14</td>
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<td>Body Weight (g)</td>
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<td>41.33 ± 0.54</td>
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<td>LV Mass (g)</td>
<td>0.16 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.22 ± 0.02</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>LV Mass/BW</td>
<td>0.60 ± 0.01</td>
<td>0.59 ± 0.01</td>
<td>0.54 ± 0.07</td>
<td>0.57 ± 0.04</td>
</tr>
</tbody>
</table>

In vivo cardiac function and ventricular wall measurements were assessed via ultrasound echocardiography (VisualSonics Vevo 770) in isoflurane anesthetized WT and MCD LF and DIO mice (n = 5-12). Values represent means ± SE. *P<0.05, indicates a significant difference from LF counterpart. †P<0.05, indicates a significant difference from WT (DIO). LF = low fat, DIO = diet induced obesity, LVPW = left ventricular posterior wall, LVID = left ventricular internal diameter, d = diastole, s = systole.
Table 3: MCD deficiency results in an improved myocardial insulin sensitivity index following DIO

<table>
<thead>
<tr>
<th></th>
<th>WT (LF)</th>
<th>MCD-/- (LF)</th>
<th>WT (DIO)</th>
<th>MCD-/- (DIO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin sensitivity</td>
<td>734 ± 125</td>
<td>1351 ± 23</td>
<td>167 ± 31</td>
<td>554 ± 82*</td>
</tr>
</tbody>
</table>

Insulin sensitivity index was calculated as the absolute increase (Δ[with insulin – without insulin]) in glucose oxidation (nmol/g dry weight/min) in response to insulin (100 µU/mL) treatment in hearts obtained from WT and MCD-/- mice (n = 5-7). Values represent means ± SE. *P<0.05, indicates a significant difference from WT (DIO). LF = Low fat, DIO = Diet induced obesity.

Table 4: Insulin-stimulated cardiac acetyl CoA production in WT and MCD-/- mice following DIO

<table>
<thead>
<tr>
<th></th>
<th>WT (LF)</th>
<th>MCD-/- (LF)</th>
<th>WT (DIO)</th>
<th>MCD-/- (DIO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl CoA Production from Glucose Oxidation (nmol/g dry weight/min)</td>
<td>2491 ± 397</td>
<td>4023 ± 181</td>
<td>811 ± 71*</td>
<td>1888 ± 159*†</td>
</tr>
<tr>
<td>Acetyl CoA Production from Palmitate Oxidation (nmol/g dry weight/min)</td>
<td>1868 ± 567</td>
<td>1629 ± 284</td>
<td>2471 ± 252</td>
<td>1866 ± 104</td>
</tr>
<tr>
<td>Total Acetyl CoA Production (Glucose + Palmitate) (nmol/g dry weight/min)</td>
<td>4522 ± 464</td>
<td>5652 ± 416</td>
<td>3282 ± 216*</td>
<td>3702 ± 192*</td>
</tr>
</tbody>
</table>

Insulin-stimulated cardiac acetyl CoA production was determined by multiplying the glucose oxidation rate by 2 and the palmitate oxidation rate by 8. These values were used to determine the overall percent contribution to TCA cycle acetyl CoA production in hearts obtained from WT and MCD-/- mice (n = 5-7). Values represent means ± SE. *P<0.05, indicates a significant difference from LF counterpart. †P<0.05, indicates a significant difference from WT (DIO). LF = Low fat, DIO = Diet induced obesity.

Table 5: Myocardial lactate and pyruvate content in WT and MCD LF and DIO mice

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Lactate</th>
<th>Pyruvate</th>
<th>Lactate + Pyruvate</th>
<th>Lactate/Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (LF)</td>
<td>223.1 ± 27.6</td>
<td>8.0 ± 3.3</td>
<td>231.2 ± 29.5</td>
<td>43.9 ± 12.1</td>
</tr>
<tr>
<td>MCD-/- (LF)</td>
<td>255.9 ± 46.6</td>
<td>4.6 ± 0.9</td>
<td>260.5 ± 46.6</td>
<td>71.8 ± 18.3</td>
</tr>
<tr>
<td>WT (DIO)</td>
<td>215.5 ± 18.5</td>
<td>6.6 ± 1.0</td>
<td>221.1 ± 19.1</td>
<td>35.8 ± 6.4</td>
</tr>
<tr>
<td>MCD-/- (DIO)</td>
<td>172.4 ± 23.4</td>
<td>25.4 ± 11.7</td>
<td>197.8 ± 22.8</td>
<td>17.7 ± 10.0</td>
</tr>
</tbody>
</table>

Lactate and pyruvate content (nmol/mg protein) were assessed during the dark cycle in the fed state in hearts obtained from WT or MCD-/- mice on either a low fat diet or subjected to DIO (n = 5-11). Values represent means ± SE. LF = Low fat, DIO = Diet induced obesity.
**Figure 1**: MCD deficiency improves insulin-stimulated glucose oxidation in DIO mice

A: DIO leads to an impairment in insulin-stimulated glucose oxidation in hearts from WT mice, which was prevented in hearts from MCD-/- mice. B: Rates of fatty acid oxidation did not differ in hearts from WT and MCD-/- mice. C: However, the contribution of myocardial fatty acid oxidation rates to acetyl CoA production was decreased in MCD-/- DIO versus WT DIO mice. Values represent mean ± SE (n = 5-7). Differences were determined using a repeated measures ANOVA. *P<0.05, significantly different from low fat counterpart. †P<0.05, significantly different from – insulin counterpart. ‡P<0.05, significantly different from + insulin high fat WT.

**Figure 2**: MCD deficiency improves PDH activity in DIO mice despite an increased expression of PDK4

A: MCD-/- DIO mice had an increased expression of PPAR target gene, PDK4. B: The active portion of the PDH complex was higher in MCD-/- DIO mice. C: Total PDH activity was also higher in MCD-/- DIO mice. D: However, the % active PDH did not differ between MCD-/- DIO and WT DIO mice. Values represent mean ± SE (n = 6). Differences were determined using Student’s two-tailed t-test. *P<0.05, significantly different from WT DIO mice.

**Figure 3**: MCD deficiency increases malonyl CoA levels in DIO mice

Malonyl CoA levels were higher in MCD-/- mice following low fat diet or DIO. Values represent mean ± SE (n = 4-5). Differences were determined using a 2-way ANOVA followed by Bonferroni post-hoc analysis. *P<0.05, significantly different from diet-matched WT mice.

**Figure 4**: MCD deficiency decreases the accumulation of fatty acid oxidative intermediates in DIO mice without decreasing TCA cycle intermediates.

MCD -/- subjected to DIO had less accumulation of myocardial A: C12-C14 acylcarnitines, B: C16-C18 acylcarnitines C: C20-C22 acylcarnitines D: Total long chain acylcarnitines compared to WT DIO mice. E: MCD-/- mice subjected to DIO did not alter myocardial short chain CoA compared to WT DIO mice. F: MCD-/- mice subjected to DIO did not have alterations in myocardial TCA cycle intermediates compared to WT DIO mice. Values represent mean ± SE (n = 5-11). Differences were determined using a 2-way ANOVA followed by Bonferroni post-hoc analysis. *P<0.05, significantly different from WT low fat fed. †P<0.05, significantly different from WT DIO.

**Figure 5**: Improvement of insulin-sensitivity in MCD -/- mice subjected to DIO does not correlate with the accumulation of myocardial lipid intermediates.

A: DIO increased long chain acyl CoAs to similar extents in both WT and MCD-/- mice. B: TG only accumulated in MCD-/- mice following DIO, but did not accumulate in WT mice. C: Ceramides do not accumulate following DIO in hearts from WT and MCD-/- mice. Values represent mean ± SE (n = 4-11). Differences were determined using a 2-way ANOVA followed by Bonferroni post-hoc analysis. *P<0.05, significantly different from low fat fed counterpart.

**Figure 6**: Neither DIO or MCD deficiency alters the expression of proteins involved in energy metabolism and mitochondrial function.

A: PGC1α expression is not altered by DIO or MCD deficiency, B: UCP2 expression is not altered by DIO or MCD deficiency. Values represent mean ± SE (n = 4 per group).

**Figure 7**: Insulin stimulated Akt phosphorylation is increased in hearts from MCD-/- mice following DIO

A: Insulin stimulated Akt phosphorylation at serine 473 is increased in hearts of MCD-/- mice subjected to DIO, B: Insulin-stimulated GSK3β phosphorylation at serine 9 in WT and MCD-/- mice subjected to DIO, C: AMPK phosphorylation is not altered in MCD-/- mice following DIO.
Values represent mean ± SE (n = 6 per group). Differences were determined using Student’s two-tailed t-test. *$P<0.05$, significantly different from WT DIO mice.

Figure 1
Figure 2

A

B

C

D

Myocardial triglycerides and glucose oxidation
Figure 3

Malonyl CoA

nmol/g wet weight

Low Fat  High Fat

Wild Type  MCD-/-
Figure 4

A  C12-C14 Acylcarnitines

B  C16-C18 Acylcarnitines

C  C20-C22 Acylcarnitines

D  Total Long Chain Acylcarnitines

E  Short Chain Acyl CoA

F  Organic Acids

Myocardial triglycerides and glucose oxidation
Figure 5

A. Long Chain Acyl CoA

B. Triglycerides

C. Ceramides