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Original article

Cardiac-specific deficiency of the mitochondrial calcium uniporter augments fatty acid oxidation and functional reserve

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ABSTRACT

The mitochondrial calcium uniporter (MCU) relays cytosolic Ca^{2+} transients to the mitochondria. We examined whether energy metabolism was compromised in hearts from mice with a cardiac-specific deficiency of MCU subjected to an isoproterenol (ISO) challenge. Surprisingly, isolated working hearts from cardiac MCU-deficient mice showed higher cardiac work, both in the presence or absence of ISO. These hearts were not energy-starved, with ISO inducing a similar increase in glucose oxidation rates compared to control hearts, but a greater increase in fatty acid oxidation rates. This correlated with lower levels of the fatty acid oxidation inhibitor malonyl CoA, and to an increased stimulatory acetylation of its degrading enzyme malonyl CoA decarboxylase and of the fatty acid β-oxidation enzyme β-hydroxyacyl CoA dehydrogenase. We conclude that impaired mitochondrial Ca^{2+} uptake does not compromise cardiac energetics due to a compensatory stimulation of fatty acid oxidation that provides a higher energy reserve during acute adrenergic stress.

1. Introduction

Mitochondrial uptake of calcium (Ca^{2+}) in vertebrates has been documented since the early 1960s [1]. This process has a central role in cell physiology by stimulating ATP production, shaping cytosolic Ca^{2+} transients, and regulating cell death [2]. Mitochondrial Ca^{2+} uptake has been attributed to a putative mitochondrial Ca^{2+} uniporter (MCU), with the channel eventually being identified in 2011 [3,4]. The gene for MCU contains a sequence coding for two transmembrane helices forming two domains embedded in the inner mitochondrial membrane and are linked by a highly conserved amino acid sequence facing the intermembrane space [3,4]. In addition, several other proteins associated with this channel have been identified as regulatory components [5–8].

Early studies examining the metabolic effects of increasing heart work showed an increased myocardial glucose uptake and glucose oxidation with epinephrine treatment [9]. This increase in glucose oxidation rates is associated with increased glycolysis and enhanced pyruvate dehydrogenase (PDH) activity even in the presence of high fatty acid levels [10,11]. This was mainly attributed to the stimulatory effect of an increase in mitochondrial Ca^{2+} on PDH phosphatase (PDHP) [12]. While increasing workload also increases fatty acid oxidation, this occurs with a lower magnitude than glucose oxidation (glycogenolysis and PDH stimulation) [10,13]. This presumably occurs due to adrenergic-induced increases in cytosolic Ca^{2+} that are relayed into mitochondria by the MCU, and hence influence the activity of the

Abbreviations: ACC, acetyl CoA carboxylase; AMPK, 5′ AMP-activated protein kinase; CoA, coenzyme A; CPT, carnitine palmitoyltransferase; CS, citrate synthase; ETC, electron transport chain; FOXO1, forkhead box protein O1; GCN5-L1, general control of amino acid synthesis 5- like enzyme 1; ISO, isoproterenol; LCAD, long-chain acyl CoA dehydrogenase; LVDP, left ventricular developed pressure; MCD, malonyl CoA decarboxylase; MCU, mitochondrial calcium uniporter; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NCLX, sodium-calcium exchanger; PDH, pyruvate dehydrogenase; PDHP, pyruvate dehydrogenase phosphatase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PTM, post-translational modifications; SIRT, sirtuin; TCA, tricarboxylic acid; UPLC, ultra-performance liquid chromatography; VDAC, voltage-dependent anion channel; β-HAD, β-hydroxyacyl CoA dehydrogenase

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intramitochondrial Ca^{2+}-sensitive dehydrogenases and PDHP [11].

Mitochondria derived from MCU KO ($MCU^{-/-}$) mice have no apparent capacity to rapidly uptake Ca^{2+} [14,15]. Despite this, basal metabolism in the skeletal muscle of $MCU^{-/-}$ mice seems unaffected despite alterations in the phosphorylation and activity of PDH [14]. In addition, $MCU^{-/-}$ mice exhibit marked impairment in their ability to perform strenuous work and respond to acute exercise stimulation [14,15]. However, it is not clear what effect increasing workload has on cardiac function and energetics if cardiac mitochondrial Ca^{2+} uptake is compromised. Therefore, we conducted our study on cardiac-specific and inducible MCU-deficient mice hypothesizing that subjecting their hearts to an increased workload (*i.e.*, isoproterenol stress) will result in impaired glucose oxidation and reduced efficiency due to a higher phosphorylation status of PDH.

2. Results

2.1. MCU-deficient hearts show enhanced cardiac function at comparable heart rates

The generation of cardiac-specific MCU-deficient mouse ($MCU^{fl/fl\text{-}MCM}$), which display a normal healthy phenotype along with the accompanying impairment of mitochondrial Ca^{2+} uptake, was previously described [15]. To evaluate functional consequences of myocardial MCU deficiency, we investigated several functional parameters before (basal) and after the addition of insulin (100 μU/ml), as well as after the introduction of ISO (10 nM) in $MCU^{fl/fl\text{-}MCM}$ and in $MCU^{fl/fl}$ control hearts. ISO creates a considerably increased workload representing an acute stress challenge to these hearts. In this regard, we utilized a unique *ex vivo* approach that provides an actual workload to the mouse heart through a perfusion system offering normal directional (antegrade) aortic and coronary flow of a perfusate containing physiologically relevant concentrations of energy substrates. The system also offers the ability to pump against actual afterloads and provides homeostatic and cardiac work data in parallel with ongoing assessment of energy metabolism.

In addition to previous description of the cardiac-specific MCU-deficient ($MCU^{fl/fl\text{-}MCM}$) mice [15], we confirmed here the MCU deficiency by immunoblotting. There was a significant drop in MCU protein levels in the MCU-deficient hearts to below 40% of control levels (Fig. 1A). In accordance with our previous experience with *ex vivo* working heart perfusions, we did not see changes in cardiac function upon the addition of insulin only. Henceforth, our functional assessment and statistical tests were based on pre- and post- ISO perfusions.

Despite the rapid increase of heart rate after administration of ISO in all hearts, there was no statistical difference between the two genotypes throughout the perfusion protocol (Fig. 1B). However, and in contrast to our expectations, $MCU^{fl/fl\text{-}MCM}$ hearts showed an enhanced cardiac function throughout the protocol even at baseline. This is displayed as higher left ventricular developed pressure (LVDP), cardiac output, peak systolic pressure (PSP), as well as cardiac work (Fig. 1C–F). Likewise, the acute initial response to ISO showed a generally similar trend (Supplementary Fig. 2). In summary, despite a decrease of cardiac MCU protein, hearts showed an enhanced cardiac function that persisted after introducing the ISO challenge.

2.2. Fatty acid oxidation in MCU-deficient hearts is increased in response to isoproterenol stress

To investigate the energy substrate preference in hearts with impaired MCU function, we utilized a radioisotope-based method tracing the oxidation of fatty acids and glucose, the two major energy substrates for the heart. This method provides an actual quantitative assessment of the contribution of fatty acid and glucose oxidation to the tricarboxylic acid (TCA) cycle over the time course of perfusion. Unexpectedly, the basal (w/o insulin) metabolic rates in $MCU^{fl/fl\text{-}MCM}$ hearts of both fatty acid oxidation and glucose oxidation were comparable to controls rates (Fig. 2A & B). This suggests that the decrease in mitochondrial Ca^{2+} uptake in MCU-deficient hearts is not impairing flux through the TCA cycle dehydrogenases (isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) under basal conditions in accordance with previous reports that have used a similar animal model [15,16]. As expected, addition of insulin increased glucose oxidation (Fig. 2A) and decreased fatty acid oxidation (Fig. 2B) in the control hearts. Interestingly, in the presence of insulin MCU-deficient hearts showed an unexpected enhanced response in the rate of glucose oxidation as compared to controls (Fig. 2A). This suggests that MCU deficiency was not decreasing PDH activity (the rate-limiting enzyme for glucose oxidation) due to a decrease in Ca^{2+}-activation of PDHP. The decline in fatty acid oxidation rates seen in the presence of insulin also occurred in the MCU-deficient hearts, with no statistical difference between the two genotypes (Fig. 2B). These responses disagree with previous suggestions of a correlation between MCU deficiency and metabolic inflexibility [17]. Although glucose oxidation was enhanced in both groups upon the administration of ISO, only the $MCU^{fl/fl\text{-}MCM}$ hearts interestingly showed accelerated fatty acid oxidation rates that were unmatched by the controls (Fig. 2A & B). This translated into a higher contribution of fatty acid oxidation to TCA cycle acetyl CoA production in MCU-deficient hearts compared to controls (Fig. 2C). It is important here to note that cardiac efficiency was not different between the two animal groups at any specific treatment and tended to decrease with ISO treatment (Fig. 2D). As the hearts subjected to ISO struggle to produce sufficient magnitude of cardiac work while consuming significant amounts of energy substrates, this leads to a decreased efficiency compared to pre-ISO [18].

The increase in fatty acid oxidation in MCU-deficient hearts in response to ISO was associated with lower levels of malonyl CoA (Fig. 2E). Malonyl CoA is a potent biological inhibitor of carnitine palmitoyltransferase I (CPT I), the gatekeeper of mitochondrial fatty acid uptake and subsequent oxidation [19]. The accelerated fatty acid oxidation was also correlated with improved energy reserve as shown by a higher acetyl CoA/CoA ratio in the MCU-deficient hearts (Fig. 2F). Therefore, MCU-deficient hearts did not show compromised energy production or metabolic inflexibility in the absence or presence of insulin. Rather, the higher cardiac work displayed by these hearts correlated well with enhanced energy production and reserve, with a greater reliance on fatty acid oxidation in response to ISO stimulation.

2.3. Protein levels and phosphorylation of key components of energy metabolism in MCU-deficient hearts display minor variation

Since myocardial MCU deficiency was not associated with poor cardiac function or restricted energy metabolism, we aimed at explaining these unexpected findings by investigating important proteins involved in myocardial metabolism. We conducted Western blotting experiments to investigate the protein expression and phosphorylation of key important components of cardiac energy substrate metabolism. It is worth mentioning here that all of the presented molecular data were obtained from heart tissue freeze-clamped at the end the *ex vivo* perfusion protocols and thus is largely represents the last phase of perfusion (insulin + ISO). There were no significant changes seen in the phosphorylation of PDH, AMP-activated protein kinase (AMPK), or acetyl CoA carboxylase (ACC) (Fig. 3A & B). Similarly, protein levels of malonyl CoA decarboxylase (MCD), responsible of malonyl CoA degradation, and those of the β-oxidation enzymes long-chain acyl-CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were not different (Fig. 3C & D) between the experimental groups.

It is unlikely that the metabolic differences seen in MCU-deficient mouse hearts are a result of differences in mitochondrial content. Although we did not directly assess the mitochondrial content in this study, few surrogate indicative points were tested and showed no

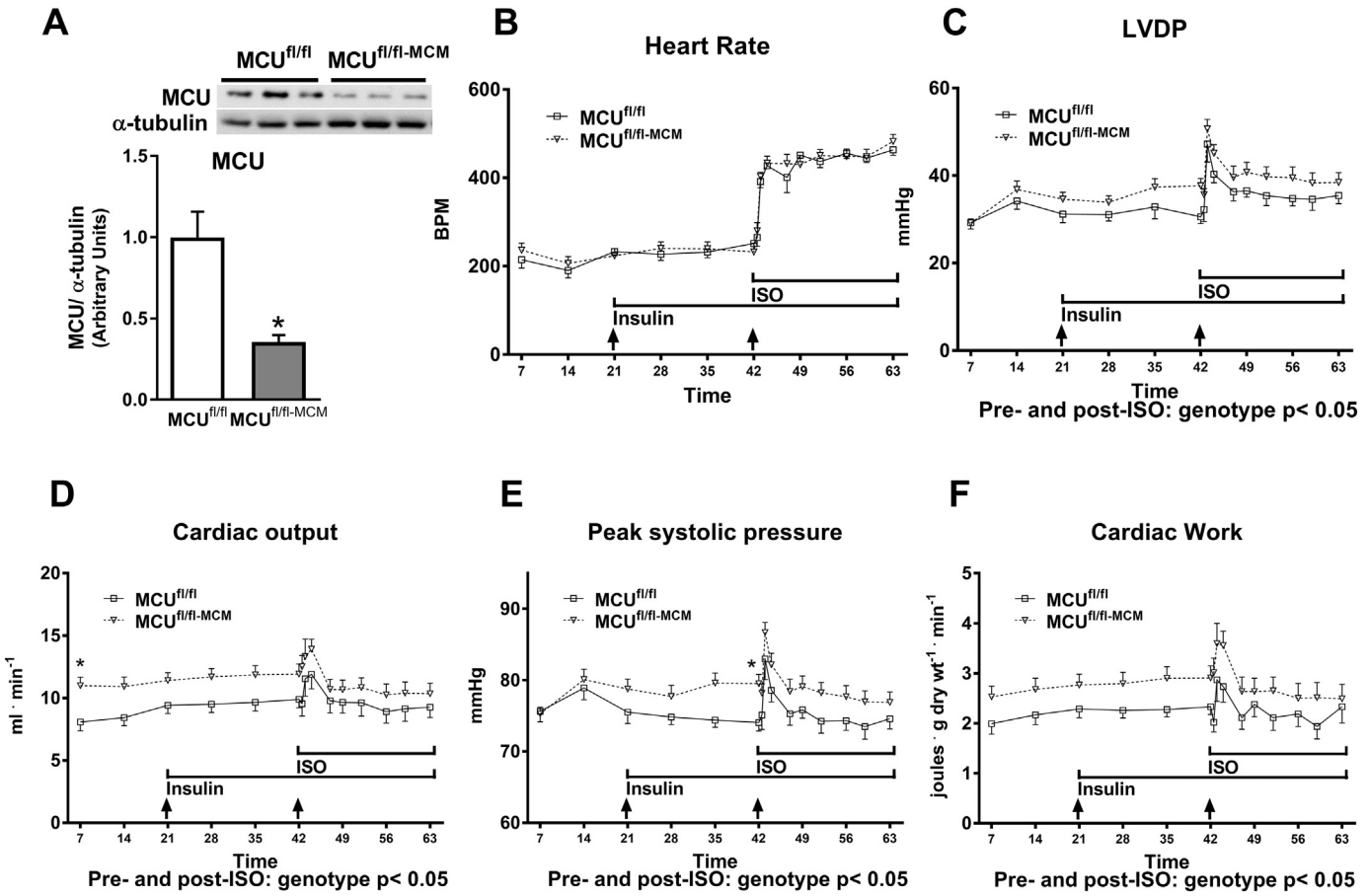

Fig. 1. MCU deficient hearts show enhanced cardiac function at comparable heart rates. (A) Western blotting confirmation of MCU protein deficiency in $MCU^{fl/fl\text{-}MCM}$ mouse hearts compared to $MCU^{fl/fl}$ control after 8-week tamoxifen treatment. α-tubulin was used as a protein loading control. *P < 0.05 versus $MCU^{fl/fl}$ control (n = 4-6). Functional parameters of 63 minutes *ex vivo* working heart perfusions of $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$ hearts showing (B) heart rate, (C) left ventricular developed pressure (LVDP), (D) cardiac output (CO), (E) peak systolic pressure (PSP), and (F) cardiac work. Insulin (100 μU/ml) was added at 21 min, followed by addition of ISO (10nM) at 42 min as described in methods. Cardiac functional parameters data (B to E) were recorded using an MP100 system from AcqKnowledge (BIOPAC Systems, Inc.), while cardiac work was calculated as explained in experimental procedures. Statistical significance over the time course of pre- and post- ISO between $MCU^{fl/fl\text{-}MCM}$ and $MCU^{fl/fl}$ control in B to F was determined using 2-way ANOVA with repeated measures and, if significant, is indicated below the specific functional figure. For data in B-F, n = 10-13, *P < 0.05 compared to $MCU^{fl/fl}$ at the specific time point using Bonferroni post-hoc test. All values represented as mean ± SEM. *Abbreviations*: MCU, mitochondrial calcium uniporter; ISO, isoproterenol.

differences including levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and the voltage-dependent anion channel (VDAC) (Fig. 3C & D), as well as citrate synthase (CS) (Supplemental Fig. 1A & B), which are related to mitochondrial biogenesis and abundance. Moreover, two previous studies using the same mouse model did not report a difference in mitochondrial content in the knockout hearts compared to controls [15,16]. In addition, total PDH and FOXO1 protein, associated with glucose oxidation and insulin signaling pathways [18,20] was not different between MCU-deficient hearts and $MCU^{fl/fl}$ controls (Supplementary Fig. 1A and B).

As previously reported [15], protein levels of mitochondrial sodium-calcium exchanger (NCLX) showed a compensatory decrease which paralleled the partial deletion of MCU in $MCU^{fl/fl\text{-}MCM}$ hearts (Supplementary Fig. 1A & B). Interestingly, both electron transport chain (ETC) complex I and II protein levels were decreased in $MCU^{fl/fl\text{-}MCM}$ (Fig. 3C & D). However, the decline appears to be mild and is unlikely impeding oxidative phosphorylation processes based on the unrestricted metabolic rates discussed above. In summary, the protein levels and phosphorylation of several components of cardiac energy metabolic pathways appear unaltered in MCU-deficient hearts, with the exception of mild decrease in ETC complex I and II protein levels.

2.4. Protein acetylation is altered in MCU-deficient hearts

As complex I and II protein content was decreased in MCU-deficient hearts, we hypothesized that NAD^+/NADH turnover could be affected. Therefore, we measured NAD^+ and NADH levels and the ratio between the oxidized to reduced NAD forms. The NAD^+/NADH ratio has important implications on the activity of sirtuins which are NAD^+-dependent deacylases distributed among cellular compartments and involved in lysine deacylation processes. Post-translational modifications (PTM) involving acetylation are known to regulate cellular energy metabolism [21]. NAD^+ levels were significantly lower, whereas NADH content in MCU-deficient hearts was not different than controls (Fig. 4A). Consequently, the NAD^+/NADH ratio was lower in $MCU^{fl/fl\text{-}MCM}$ hearts compared to $MCU^{fl/fl}$ (Fig. 4A).

Since decreased NAD^+/NADH ratio, such as seen with Complex I deficiency, affects sirtuins activity [22], we examined what effect MCU deficiency has on total cardiac lysine acetylation and of specific key metabolic proteins/enzymes. Although total protein acetylation (Fig. 4B) and the protein levels of SIRT1, SIRT3, and SIRT4 (nuclear and mitochondrial deacetylases), as well as GCN5L1 (mitochondrial acetyltransferase), were not different from controls (Fig. 4C & D), the acetylation status of β-HAD (a fatty acid β-oxidation enzyme) was increased, which we have previously shown to increase fatty acid

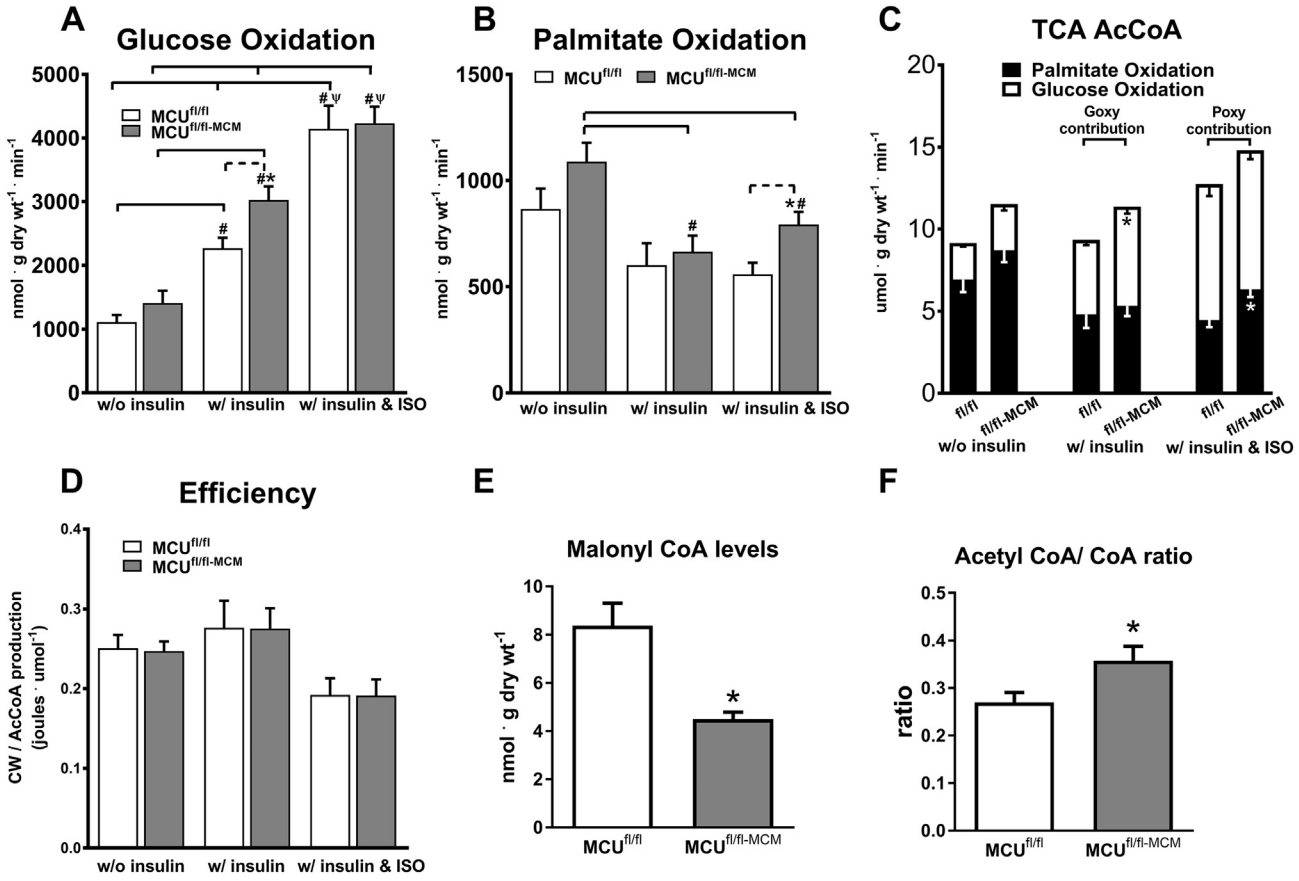

Fig. 2. MCU deficient heart metabolism is altered in response to isoproterenol stress. Energy substrate metabolic rates during *ex vivo* working heart perfusions of $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$ showing (A) glucose oxidation, and (B) palmitate oxidation rates at baseline (21 min), with insulin (100 μU/ml) (21 min), and after ISO (10nM) administration (21 min). (C) Specific contributions of oxidized palmitate versus glucose in $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$ hearts in the production of TCA cycle acetyl-CoA during the three different perfusion periods mentioned in A and B. (D) Cardiac efficiency of $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$ calculated as the cardiac work normalized for total TCA cycle acetyl-CoA production during the three different perfusion periods mentioned in A and B. (E) Malonyl-CoA levels, expressed as nmol per g dry weight of tissue, and (F) Acetyl-CoA/CoA ratio measured in samples from heart ventricles freeze-clamped at the end of the 63 min heart perfusion protocol of $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$. *$P < 0.05$ versus $MCU^{fl/fl}$ control at the same treatment period (unpaired t-test). #$P < 0.05$ versus baseline, ψ$P < 0.05$ versus 'w/ insulin' of same genotype group, 1-way ANOVA with Bonferroni's post hoc test (n = 8-13). All values represented as mean ± SEM. *Abbreviations*: Goxy, glucose oxidation; Poxy, palmitate oxidation; MCU, mitochondrial calcium uniporter; ISO, isoproterenol; TCA, tricarboxylic acid; CoA, coenzyme A.

oxidation rates [23,24], although LCAD acetylation was not altered (Fig. 4E & F). To test if this hyperacetylation status of β-HAD is associated with enhanced activity of this enzyme, we measured β-HAD enzymatic activity which was increased in accordance with its acetylation status (Fig. 4G). Acetylation of MCD (involved in malonyl CoA degradation) was also increased (Fig. 4E & F), which is has been associated with increases in its enzymatic activity [25] and may therefore explain the decrease in malonyl CoA we observed. PGC-1α (a target of SIRT1, Supplementary Fig. 1C&D) was also increased in $MCU^{fl/fl\text{-}MCM}$ hearts. This was not seen with PDH (Supplementary Fig. 1C & D). These data suggest that increased acetylation of β-HAD and MCD (resulting in a decrease in malonyl CoA levels) may be responsible for the increase in fatty acid oxidation seen in the MCU-deficient hearts.

3. Discussion and conclusions

The heart has a large energy demand in order to maintain continuous contractile function which further intensifies during fight or flight stressful situations [15,18]. A rise in mitochondrial Ca^{2+} is believed to actively contribute to the increase in energy production needed to sustain contractile function following adrenergic stimulation by increasing the activity of mitochondrial TCA cycle dehydrogenases and PDH (secondary to activation of PDHP) [26,27]. Since Ca^{2+} crosses the inner mitochondrial membrane primarily through the MCU channel [3], a deficiency in MCU would be expected to decrease energy production and compromise contractile function of the heart, especially in response to an adrenergic stimulation. However, we directly demonstrate that MCU deficiency does not compromise energy production or contractile function in the heart. This is primarily due to a compensatory increase in fatty acid oxidation and higher energy reserve in the heart.

Of interest, we demonstrate a higher than normal cardiac work in mice with a cardiac specific MCU deficiency, even after the induction of an ISO stress. Although this finding appears unexpected, this increased work was probably the result of a compensatory increase in energy production originating from fatty acid oxidation. We have utilized an *ex vivo* model as opposed to *in vivo* models used in previous studies which mostly demonstrated that MCU deletion suppresses cardiac responsiveness to beta-adrenergic stimulation [15,16,28]. However, we believe that our results are important in that they highlight the direct response in isolation from whole-body adrenergic response and provide distinct evidence that these mice deficient in myocardial MCU at least do not show impairment in their cardiac response to ISO. We further demonstrate these findings in Supplementary Fig. 2 by focusing on the acute response within the first few minutes of ISO administration. Although the initial response in some functional parameters including heart rate and aortic outflow was not different between MCU-deficient hearts and their controls (Supplementary Fig. 2A and E), other *ex vivo*

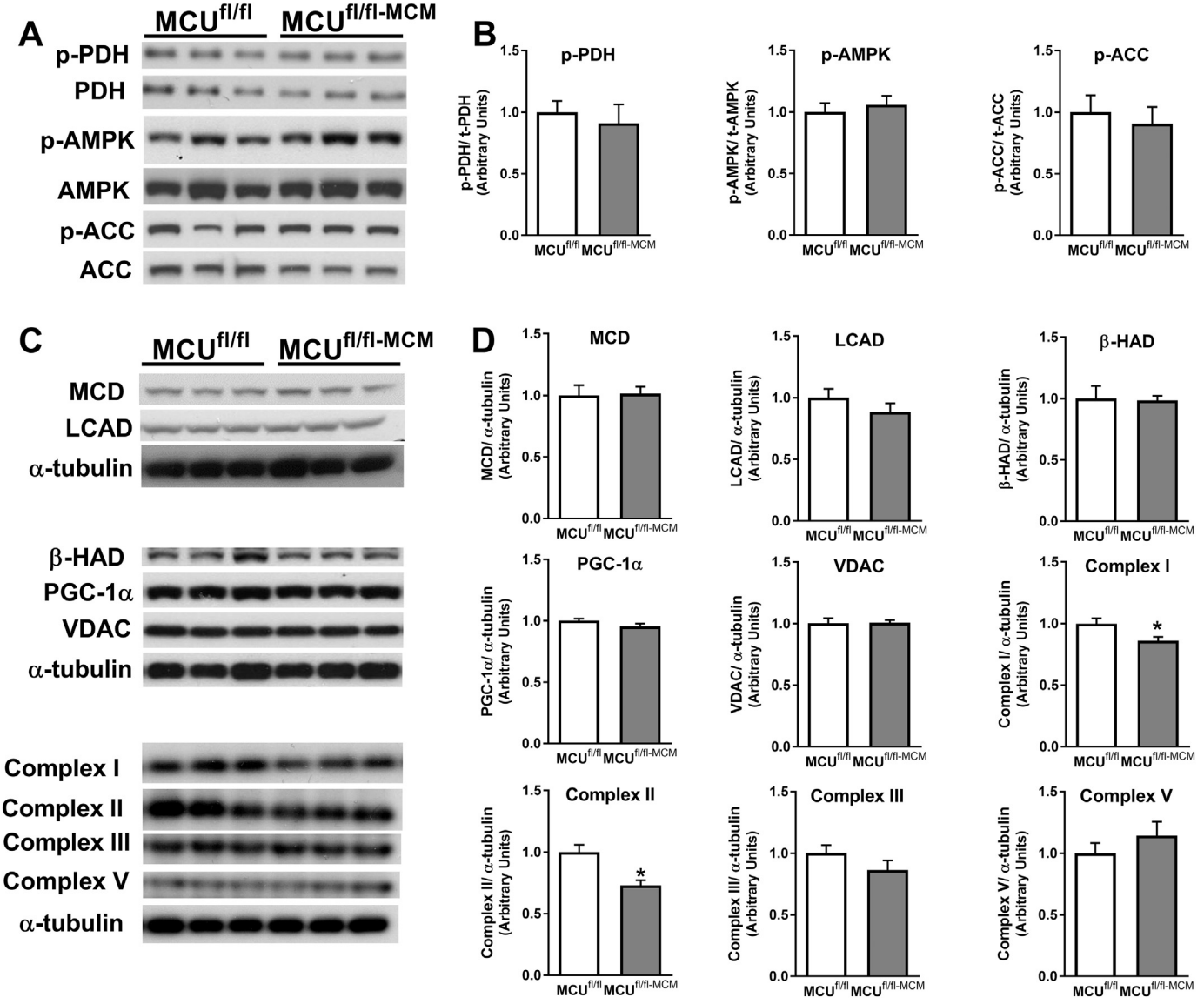

Fig. 3. Protein levels and phosphorylation of key components of energy metabolism in MCU deficient hearts display minor variation. (A) Western blotting analysis of the phosphorylation of key enzymes involved glucose and fatty acid oxidation, S293 p-PDH, T172 p-AMPK, and S79/1200 p-ACC, which is associated with their activity. Each phosphorylated form of these enzymes is normalized for the total protein level of the respective enzyme. (B) Quantification of data presented in (A). (C) Western blotting analysis of the protein levels of key enzymes and proteins involved in energy metabolism and oxidative phosphorylation as well as mitochondria biogenesis. Blots of the following proteins are presented: MCD; LCAD; β-HAD; PGC-1α; VDAC; ETC complexes I, II, III, and V. α-tubulin was used as a protein loading control. (D) Quantification of data presented in (C). WB samples were made from heart ventricles freeze-clamped at the end of the 63 min heart perfusion protocol of $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$. *$P < 0.05$ versus $MCU^{fl/fl}$, (n = 5-6). All values represented as mean ± SEM. *Abbreviations*: PDH, pyruvate dehydrogenase; AMPK, 5' AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; MCD, malonyl-CoA decarboxylase; LCAD, long-chain acyl CoA dehydrogenase; β-HAD, β-hydroxyacyl CoA dehydrogenase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; VDAC, voltage-dependent anion channel; ETC, electron transport chain.

functional parameters showed higher value in $MCU^{fl/fl\text{-}MCM}$ hearts with regard to their early response to ISO including PSP, LVDP, cardiac output, and coronary flow (Supplementary Fig. 2B–D and F). Particularly, largely enhanced coronary flow in the face of an aortic outflow in $MCU^{fl/fl\text{-}MCM}$ hearts that is not different from $MCU^{fl/fl}$ controls, which may have resulted from some unknown mCa^{2+}-mediated mechanism, has contributed to the enhanced cardiac output and cardiac work. In accordance with our findings on MCU-deficient hearts, we recently reported that MCU deletion in skeletal muscles, although inhibiting acute mitochondrial Ca^{2+} influx, leads to an enhancement of muscle endurance and performance under conditions of fatigue [29], a state of enhanced physiological demands akin to persistence ISO stimulation to $MCU^{fl/fl\text{-}MCM}$. This was also interestingly accompanied by a preferential shift towards fatty acid oxidation for energy production [29], which further agrees with our current report on heart-specific MCU deficiency.

We have utilized the $MCU^{fl/fl}$ mice as our control. While the lack of inclusion of MCU^{MCM} mice as an additional control group may have constituted a limitation to our design, this has resulted from the fact that we had a significant shortage in MCU^{MCM} mice (only $n = 2$) due to technical and procedural barriers thus preventing us from obtaining sufficient results to be included in the study. In this regard, although the tamoxifen- induced MerCreMer (MCU^{MCM}) mouse model has been suggested by previous studies to associate with transient cardiac myopathy and dysfunction [30–32], this was seen in all Cre-positive mice including those cross-bred with loxP mice, which in our study are represented by the $MCU^{fl/fl\text{-}MCM}$ experimental mice [30]. However, all

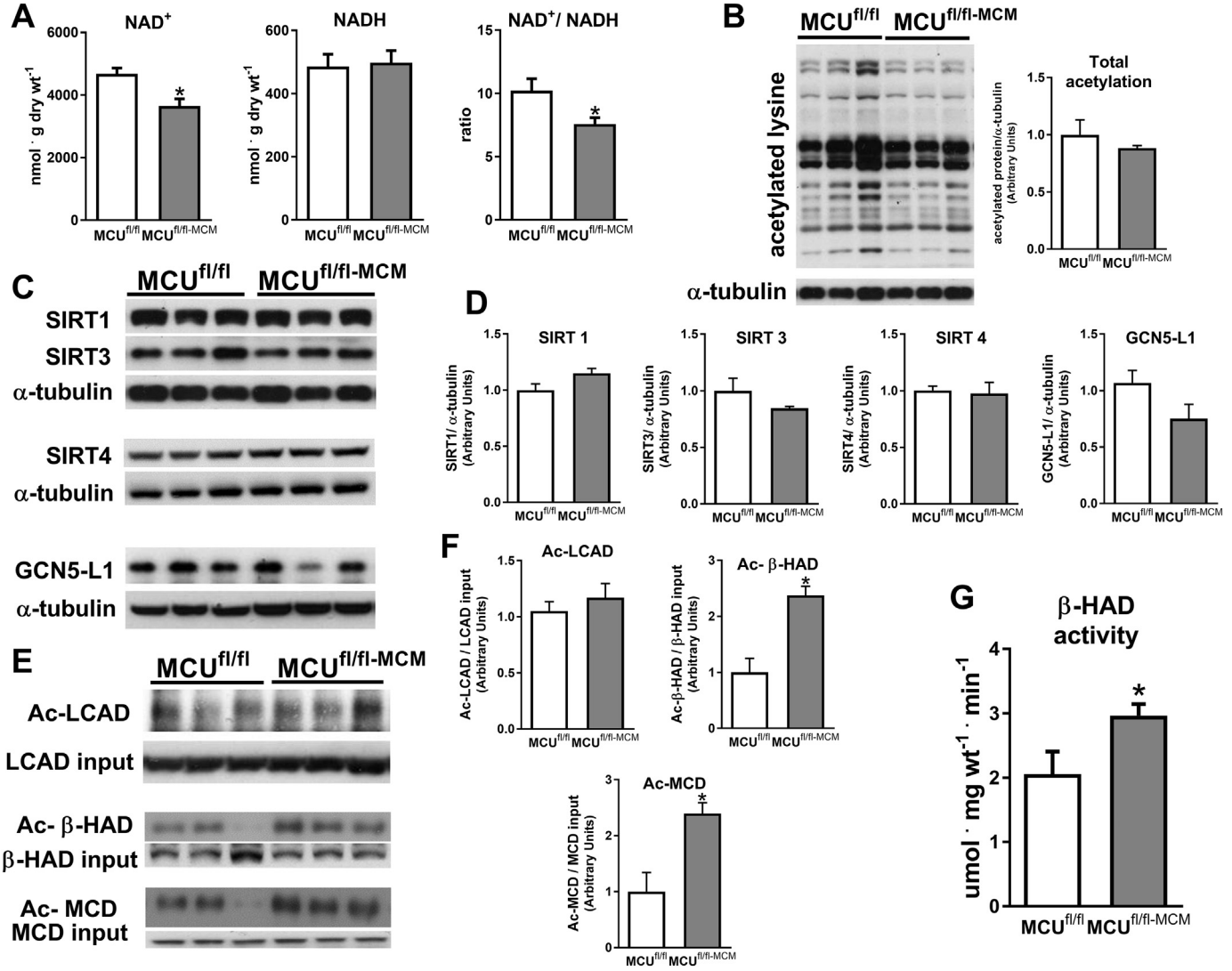

Fig. 4. Protein acetylation is altered in $MCU^{fl/fl\text{-}MCM}$ hearts. (A) NAD^+, NADH levels, expressed as nmol per g dry weight of tissue, and NAD^+/NADH ratio in heart tissue from $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$ mice (n = 10). (B) Western blotting analysis and quantification of total protein acetylation in $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$ mouse hearts. Total acetylation was detected in the same membrane with same loading control in figure 3C (β-HAD, PGC-1α and VDAC). α-tubulin was used as a protein loading control. (C) Western blotting analysis of SIRT1, SIRT3, SIRT4, and GCN5-L1, important enzymes regulating lysine acetylation of key metabolic proteins. α-tubulin was used as a protein loading control. (D) Quantification of data presented in (C). (E) Acetylation status of β-HAD, LCAD, and MCD enzymes involved in fatty acid oxidation assessed by reverse immunoprecipitation (IP) acetylation assay explained in Supplementary Material. (F) Quantification of data presented in (E) by normalizing for IP input of the respective total protein levels. (G) β-HAD enzymatic activity expressed as umol 3-hydroxyacyl-CoA produced per mg weight of tissue per minute (n = 7). NAD+, NADH, western blotting, immunoprecipitation, and β-HAD activity samples were prepared from heart ventricles freeze-clamped at the end of the 63 min heart perfusion protocol of $MCU^{fl/fl}$ and $MCU^{fl/fl\text{-}MCM}$. For data in B-F n = 4-6. *P < 0.05 versus $MCU^{fl/fl}$. All values represented as mean ± SEM. *Abbreviations*: NAD^+ and NADH, nicotinamide adenine dinucleotide oxidized and reduced forms, respectively; SIRT1, SIRT3, and SIRT4, sirtuin 1,3, and 4, respectively; GCN5-L1, general control of amino acid synthesis 5- like enzyme 1; Ac-LCAD, acetylated long-chain acyl CoA dehydrogenase; Ac- β-HAD, acetylated β-hydroxyacyl CoA dehydrogenase; Ac-MCD, acetylated malonyl-CoA decarboxylase.

those studies showed cardiac dysfunction and reduction of energy/metabolism rather than improved function and unrestrained energetics as we show here, indicating that our Cre-positive mice did not suffer from a cardiac debility. Noteworthy, those studies emphasized a role for the amount (dose) of injected tamoxifen to induce the transient cardiac phenotype [30–32]. Therefore, we used a mild tamoxifen administration protocol through the oral route (in food) to all control and knockout mice and gave a sufficient tamoxifen washout period before conducting any experiments (3 months), which normally allows avoidance of possible transient adverse effects.

The lack of a functional deficit in the MCU-deficient hearts subjected to an adrenergic stress is supported by other approaches in which MCU activity was altered. For example, ruthenium red (an inhibitor of MCU) increased cardiac function, while spermine (an opener of MCU) decreased cardiac function in hearts subjected to ischemic post- conditioning [33]. Conversely, in another study using *ex vivo* perfused mouse hearts, both Ru360 and spermine (an inhibitor and stimulator of MCU, respectively) induced negative and positive inotropic effects, respectively with antagonistic effects [34]. In this latter study, inotropic stimulation with ISO elevated oxygen consumption, increased Ca^{2+}-dependent activation of PDH, and increased mitochondrial Ca^{2+} content. These effects were abolished by Ru360, suggesting an uncoupling between workload and ATP production upon MCU inhibition [34]. It should be recognized, however, that hearts in this study were perfused in the absence of physiologically relevant concentrations of fatty acids, which would not allow for a compensatory increase in fatty acid oxidation, such as we observed in our study. Another recent study showed in whole body MCU knockout mice that although cardiomyocyte

mitochondrial matrix Ca^{2+} levels were reduced, normal levels of cardiac function, ATP, and respiratory control ratio under basal conditions were seen in addition to normal responses to ISO stress and transverse aortic constriction [35]. Other studies using myocardial MCU inhibition by transgenic expression of a dominant-negative MCU or cardiac deletion also showed normal resting heart rates despite incapability of the animals of physiological "fight or flight" heart rate acceleration [15,28].

Noteworthy, the contribution of glucose oxidation to energy production was also not compromised in MCU-deficient hearts. We also did not see a decrease in overall TCA cycle activity, suggesting that the demonstrated decrease in mitochondrial Ca^{2+} uptake associated with MCU deficiency [15,16] is not impairing either the TCA cycle dehydrogenases or PDH activity. The insulin-stimulated increases in glucose oxidation in our MCU-deficient hearts are particularly interesting. A recent study suggested a correlation between reduced levels of MCU and insulin resistance as found in type-1 diabetic mice and in mouse neonatal cardiomyocytes cultured under hyperglycemic conditions [17]. In those cells, reduced MCU levels correlated with decreased glucose oxidation associating with higher phosphorylation and lower activity of PDH. This variability in phosphorylation of PDH was not reproduced in our study perhaps as a result of the obscuring effect of ISO stimulation on PDH phosphorylation. Here again, however, MCU deficiency models have shown mixing results regarding PDH phosphorylation/activity [14,15].

While it would be ideal to measure changes in iCa^{2+} and mCa^{2+} in the presence of insulin, this was not possible in our experimental system. Dysregulation of iCa^{2+} homeostasis in conditions of mitochondrial dysfunction has been associated with the pathogenesis of insulin resistance and T2D [36], it is unclear whether insulin can directly regulate MCU activity. As for the glucose oxidation rates in the MCU-deficient hearts that were comparable to controls after ISO administration, we believe that the rates have leveled up to a maximum in all perfused hearts due to the augmented energy demands under the increased workload. Additionally, since insulin was introduced to these hearts before the addition of ISO, and since we observed expected metabolic effects with insulin alone, it is possible that insulin may have "primed the metabolic system" for further effects of ISO. It may have facilitated ISO having its metabolic effects, particularly with regard to isoproterenol increasing fatty acid oxidation in the MCU KO hearts.

The increase in fatty acid oxidation seen in the MCU-deficient hearts was likely due, in part, to a decrease in malonyl CoA levels, which would result in an increase in mitochondrial fatty acid uptake. Our data suggest that this decrease in malonyl CoA was not due to a reduction in ACC activity, but rather an increase in malonyl CoA degradation. MCD acetylation was increased in the MCU-deficient hearts (Fig. 4E & F), which is associated with stimulation of its malonyl CoA degradation activity [25]. In addition, an increased acetylation of the fatty acid β-oxidation enzyme, ß-HAD occurred (Fig. 4E & F) which is correlated with enhanced activity of fatty acid oxidation [23,24]. Therefore, alterations in acetylation of these enzymes may explain the increase in fatty acid oxidation we observed in MCU-deficient hearts. We propose that the drop in NAD^+ levels seen in the MCU-deficient hearts decreased SIRT3 activity (which deacetylates ß-HAD) and decreased SIRT4 (which deacetylates MCD), leading to an increase in ß-HAD activity and a decrease in malonyl CoA levels. It is unclear how MCU regulates cardiac metabolism at rest. Impaired MCU function could affect the at-rest heart through similar changes in malonyl CoA or fatty acid oxidative enzyme acetylation that we observed in $MCU^{fl/fl\text{-}MCM}$ hearts treated with ISO. Unfortunately, however, we were not able to measure either malonyl CoA or fatty acid oxidation enzyme acetylation in hearts at rest, since we did not have tissue from hearts perfused under these conditions.

Interestingly, a discrepancy in the ratio of NAD^+/NADH exists between findings in our study and that of Luongo et al. 2015 [16]. However, this may have stemmed from the fact that those mice, unlike ours, received a five-day tamoxifen injection protocol at 40 mg/kg/day. According to another study focusing on the consequences of tamoxifen injuctions on mice with tamoxifen inducible Cre recombinase under the control of the α-MHC promoter, this injection protocol results in a significant fall in systolic function 6 days after the initiation of tamoxifen treatment that resolves after 10 days of the last injection but is associated with persistent cardiac hypertrophy [31]. This cardiac phenotype, which was not evident in our mice, may have interfered in NAD+/NADH biology particularly after a catecholamine challenge. Additionally, the protein expression of ETC components was not similarly affected by the temporal MCU deletion protocol in Luongo et al. 2015 compared to our study. Combined, this resulted in activation in fatty acid oxidation in the MCU-deficient hearts.

In conclusion, MCU deficiency in the heart is not associated with energy starvation or decreased cardiac function. This is because fatty acid oxidation increases to meet the extra energy demand. These hearts showed improved energetic reserve. Mechanisms of metabolic alterations include enhancement of lysine acetylation of key regulatory metabolic proteins. Understanding the roles of mitochondrial Ca^{2+} uptake in heart metabolism may provide better insights into future therapeutic approaches for heart disease.

4. Materials and methods

4.1. Materials and animals

Materials used in this study are listed in the Supplementary Material file. The generation of the heart-specific MCU-deficient mouse was previously described [15]. Briefly, $MCU^{fl/fl}$ mice were generated by targeting the Mcu locus with loxP flanking exons 5 and 6. $MCU^{fl/fl}$ animals were crossed (or not, as controls) with mice expressing a tamoxifen-inducible Cre recombinase (MerCreMer) under α-myosin heavy chain promoter to produce $MCU^{fl/fl\text{-}MCM}$ animals. MCU deficiency was induced by feeding 8-week old animals with tamoxifen laden chow (Harlan, 400 mg/kg) along with $MCU^{fl/fl}$ controls [15] for 4 weeks, then analyzed 3 months later. All animal studies were approved by the University of Alberta Health Sciences Animal Welfare Committee and comply with the guidelines of the Canadian Council of Animal Care, Alberta.

4.2. Study protocol and heart perfusions

Hearts from male $MCU^{fl/fl\text{-}MCM}$ and $MCU^{fl/fl}$ control mice (20–22 week old) were perfused in an *ex vivo* working heart system to evaluate cardiac functional and metabolic changes. Mice were anesthetized using 12 mg sodium pentobarbital administered intraperitoneally then the hearts were isolated and perfused as described previously [37,38] with modification. The perfusion protocol consisted of 63 min aerobic perfusion divided into 3 consecutive periods of: 21 min without insulin, followed by 21 min with insulin (100 μU/mL), and another 21 min with both insulin and isoproterenol (10 nM), to induce a condition of increased workload on these hearts. Perfusion buffer consisted of a modified Krebs–Henseleit Bicarbonate (KHB) solution containing in mM (118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 5 glucose, 0.8 palmitate bound to 3% bovine serum albumin with trace amounts of radioactive [U−^{14}C]glucose, and [9, 10-^{3}H]palmitate. The buffer was continuously oxygenated with 95% O_2 & 5% CO_2 gas mixture. Glucose and palmitate oxidation rates were measured simultaneously by quantitative collection of $^{14}CO_2$ and ^3H_2O produced by the heart from metabolizing glucose and palmitate, respectively, and expressed as nmol per g dry weight per min [37,38].

Cardiac functional parameters were recorded using an MP100 system from AcqKnowledge (BIOPAC Systems, Inc.). Cardiac work was calculated as a function of peak systolic pressure and cardiac output. Efficiency is represented as the ratio of cardiac work to total calculated acetyl CoA production rates (from glucose and fatty acid oxidation

rates). At the end of the perfusion protocol, hearts were freeze-clamped in liquid nitrogen and stored at −80 °C for subsequent biochemical studies. The dry/wet tissue ratio was calculated and metabolic rates were normalized to the total dry mass of the heart.

4.3. Determination of short-CoA esters

Free CoA and its short esters including free CoA, malonyl-, and acetyl CoA were measured using an ultra-performance liquid chromatography (UPLC) procedure adapted from a method previously described with modification [39]. Details are explained in the Supplementary Materials.

4.4. Western blotting and acetylation assay

Protein levels in mouse hearts were assessed using immunoblotting as described previously [40]. Specific protein acetylation was determined using an immunoprecipitation (IP) assay as previously described [24] with some modifications. Details are explained in the Supplementary Materials.

4.5. NAD+ and NADH assay

NAD^+ and NADH content was assessed in tissue samples from frozen ventricles of the perfused hearts using Amplite™ Colorimetric NAD^+/NADH Ratio Assay Kit and according to the manufacturer's protocol.

4.6. β- HAD enzyme assay

βHAD activity was measured in whole lysates prepared from freeze-clamped heart ventricular tissues, as described previously [41]. Briefly, each lysate sample was placed into triplicate wells in a 96-well plate containing 150 mmol/l NADH and 50 mmol/l imidazole as an assay medium. The reaction was initiated by adding 100 mmol/l acetoacetyl CoA and NADH disappearance was monitored by kinetic measurement of the spectrophotometric absorbance at a wavelength of 340-nm over 5 min.

4.7. Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined using an unpaired *t*-test, one-way analysis of variance (ANOVA) or two-ANOVA with repeated measures, followed by Bonferroni post-hoc test as appropriate. Differences were deemed significant if $p < .05$.

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Author contribution

TRA conducted most of the experiments, analyzed their results, and wrote the paper. QGK performed acetylation experiments. GMU and AF conducted and analyzed immunoblotting experiments. JQK and JDM produced the animal model, provided technical advice and edited the paper. GDL conceived and coordinated the study and edited the paper with TRA.

Disclosure and conflicts of interest

The authors report no commercial or proprietary interest in any product or concept discussed in this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2018.12.019.

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