AMPK-Dependent Inhibitory Phosphorylation of ACC is not Essential for Maintaining Myocardial Fatty Acid Oxidation

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ABSTRACT

Rationale: The energy sensor adenosine monophosphate-activated protein kinase (AMPK) is thought to play an important role in regulating myocardial fatty acid oxidation (FAO) via its phosphorylation and inactivation of acetyl coenzyme A carboxylase (ACC). However, studies supporting this have not directly assessed whether the maintenance of FAO rates and subsequent cardiac function requires AMPK-dependent inhibitory phosphorylation of ACC.

Objective: To determine whether the prevention of AMPK-mediated inactivation of ACC influences myocardial FAO or function.

Methods and Results: A double knock-in (DKI) mouse (ACC-DKI) model was generated in which the AMPK phosphorylation sites Ser79 on ACC1 and Ser221 (Ser212 mouse) on ACC2 were mutated to prevent AMPK-dependent inhibitory phosphorylation of ACC. Hearts from ACC-DKI mice displayed a complete loss of ACC phosphorylation at the AMPK phosphorylation sites. Despite the inability of AMPK to regulate ACC activity, hearts from ACC-DKI mice displayed normal basal AMPK activation and cardiac function at both standard and elevated workloads. In agreement with the inability of AMPK in hearts from ACC-DKI mice to phosphorylate and inhibit ACC, there was a significant increase in cardiac malonyl CoA content compared to wild-type (WT) mice. However, cardiac FAO rates were comparable between WT and ACC-DKI mice at baseline, during elevated workloads and following a more stressful condition of myocardial ischemia that is known to robustly activate AMPK.

Conclusions: Our findings show AMPK-dependent inactivation of ACC is not essential for the control of myocardial FAO and subsequent cardiac function during a variety of conditions involving AMPK-independent and dependent metabolic adaptations.

Keywords: AMPK, ACC, fatty acid oxidation, myocardial metabolism

Nonstandard Abbreviations and Acronyms:
ACC Acetyl coenzyme A carboxylase
ACC-DKI ACC double knock-in
CPT carnitine palmitoyl CoA transferase
AMPK adenosine monophosphate-activated protein kinase
FAO fatty acid oxidation
ATGL adipose triglyceride lipase
FATP1 fatty acid transport protein 1
CD36 cluster of differentiation 36
PPARα peroxisome proliferator-activated receptor alpha
PKA protein kinase A
UCP3 uncoupling protein-3
INTRODUCTION

Acetyl-CoA carboxylase (ACC) is thought to play an important role in regulating fatty acid oxidation (FAO) in the heart1, 2. ACC catalyzes the conversion of acetyl CoA to malonyl CoA3. In muscle, malonyl CoA is an endogenous inhibitor of carnitine palmitoyl CoA transferase (CPT) 1, which regulates long-chain fatty acyl CoA import into the mitochondria for β-oxidation4, 5. Two isoforms of ACC exist in the heart (ACC 1 and 2)3, 6, and both ACC1/2 can be regulated by inhibitory phosphorylation by adenosine monophosphate-activated protein kinase (AMPK)7. AMPK phosphorylates ACC1/2 on serine residues (Ser79/212)7-9, leading to inhibition of ACC activity and decreased malonyl-CoA production10. Despite the role that AMPK plays in regulating ACC activity and subsequent FAO, whether or not AMPK-mediated inactivation of ACC plays an obligate role in the maintenance of myocardial FAO rates has not been tested.

To address this, we generated mutant mice with alanine knock-in mutations in both ACC1 (at Ser79) and ACC2 (at Ser212). In the liver and skeletal muscle, these mice maintain functional ACC and AMPK but express a mutant form of ACC that is no longer inhibited by AMPK phosphorylation11. Consistent with the loss of AMPK-mediated inactivation of ACC, we have previously shown that these ACC double knock-in (ACC-DKI) mice have significantly elevated ACC activity and impaired FAO in the liver11. However, the effect of preventing AMPK-mediated phosphorylation of ACC in the heart has not been investigated. Herein we show hearts from ACC-DKI mice have increased malonyl CoA levels compared to wild-type (WT) mice but their FAO rates are comparable to WT either at baseline or during elevated workloads and following myocardial ischemia; conditions known to result in AMPK-independent12, 13 and dependent metabolic adaptations14, 15, respectively. Together, these findings show that during work-induced metabolic adaptations as well as ischemia-induced AMPK activation, maintenance of myocardial FAO and subsequent cardiac function is not reliant on AMPK-dependent inhibitory phosphorylation of ACC.

METHODS

Animals.

ACC-DKI mice that express the two mutant isoforms of ACC that are resistant to inhibitory phosphorylation by AMPK were described previously11. For all experiments, mice of 20-33 weeks old were studied, littermate wild-type (WT) mice were used as controls and since there were no sex differences in the parameters measured, both male and female mice were used. The University of Alberta Institutional Animal Care and Use Committee approved all protocols involving mice. An expanded Methods section is available at the online data supplement.

Echocardiography and dobutamine stress echocardiography.

In vivo cardiac function was assessed using transthoracic echocardiography at baseline and 10 minutes after dobutamine injection as previously described16, 17.

Heart perfusions.

For metabolic measurements and ex vivo heart function, hearts were perfused in the working heart mode under normal and high workload conditions as described previously2. For ischemia-reperfusion studies, hearts were aerobically perfused for 30 minutes, or aerobically perfused for 30 minutes followed by 18 minutes of global no flow ischemia and 40 minutes of reperfusion as described previously18.
Tissue homogenization and immunoblot analysis.
Frozen hearts were ground using mortar and pestle, and tissue powder was homogenized in ice-cold lysis buffer. Tissue lysates were resolved by SDS-PAGE, and proteins were transferred onto a nitrocellulose membrane. Blotted proteins were reversibly visualized using MemCode stain (Pierce) and identified using the corresponding primary antibodies. Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). All densitometric data were corrected against total protein loading visualized via Memcode (Pierce) staining.

Malonyl CoA measurements.
Following heart perfusions, malonyl CoA was extracted from 20 to 30 mg of frozen ventricular tissue and quantified by Ultra-high performance liquid chromatography (UPLC)\textsuperscript{11, 19}

Statistical analysis.
Results are expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism software. Comparisons between two groups were made by unpaired two-tailed Student’s t-test. Comparisons between two or more groups were made by one way ANOVA and comparisons involving multiple time points are by two way ANOVA respectively. \textit{P}-values of less than 0.05 were considered statistically significant.

RESULTS
The loss of AMPK-mediated ACC phosphorylation in hearts of ACC-DKI mice occurs in the absence of any alterations in cardiac function or morphology.

Hearts from ACC-DKI mice (Fig. 1A) exhibited a lack of ACC phosphorylation on Ser79 (ACC1) and Ser212 (ACC2; Fig. 1B), confirming the lack of inhibitory phosphorylation by AMPK in the heart. This lack of AMPK effect on ACC phosphorylation was not secondary to any changes in levels of AMPK phosphorylation on Thr172 in hearts from either genotypes (Fig. 1C). Despite the loss of the AMPK-mediated ACC phosphorylation, hearts from ACC-DKI mice had normal structure and function \textit{in vivo}. In fact, hearts from ACC-DKI mice displayed no difference in gross structure (Fig. 2A), hypertrophy (Fig. 2B), ejection fraction (Fig. 2C), isovolumic relaxation time (Fig. 2D) or other parameters assessed by echocardiography (Table 1) compared to WT. Taken together, these data indicate that preventing AMPK-mediated inhibitory ACC phosphorylation does not have a detectable effect on cardiac morphology or function at baseline.

The loss of AMPK-mediated ACC phosphorylation in hearts of ACC-DKI mice does not alter myocardial fatty acid oxidation rates.

As expected with the loss of AMPK-mediated inhibitory phosphorylation of ACC, malonyl CoA levels were elevated in ACC-DKI mice compared to controls, both in the \textit{ex vivo} perfused hearts (Fig. 2E) and \textit{in vivo} frozen hearts (data not shown). Surprisingly, although we have previously shown that these ACC-DKI mice have impaired FAO rates in the liver\textsuperscript{11}, \textit{ex vivo} perfused hearts from these mice show no differences in myocardial FAO rates (Fig. 2F) compared to hearts from WT mice. This lack of effect on myocardial FAO rates is consistent with the normal function of these hearts during \textit{ex vivo} perfusion (Table 2; first two columns).

In order to determine whether there was a developmental compensation for the chronic ablation of the AMPK phosphorylation sites in ACC, we examined the expression of several proteins involved in regulating cardiac substrate metabolism. There was no significant change in the protein expression of...
known regulators of FAO, such as adipose triglyceride lipase (ATGL), cluster of differentiation 36 (CD36), fatty acid transport protein 1 (FATP1), or peroxisome proliferator-activated receptor alpha (PPARα)20-23 (Fig. 3A). Similarly, there was no change in Akt phosphorylation at both Ser473 and Thr308 sites, p38 phosphorylation at Thr180, or protein kinase A (PKA) phosphorylation at Thr197 (Fig. 3B), which are proteins involved in glucose metabolism24-26 as well as in the regulation of FAO27, 28. Despite the apparent lack of compensatory responses of important regulators of myocardial energy metabolism in ACC-DKI mice, there was a significant 3.6-fold increase of the uncoupling protein-3 (UCP3) in hearts of ACC-DKI mice compared to the WT (Fig. 3C). This is particularly important as UCP3 has been previously shown to increase FAO rates29, 30 and thus may be responsible for normal FAO rates observed in hearts from ACC-DKI mice in the absence of a disrupted AMPK-ACC signaling axis.

The loss of AMPK-mediated ACC phosphorylation in hearts of ACC-DKI mice does not alter myocardial FAO or function at elevated workloads.

Since we did not observe any changes in FAO rates in hearts from ACC-DKI mice during normal workloads, we sought to challenge the hearts with increased workload to ascertain their ability to adapt to an acute increase in energy demand. To do this, hearts from WT and ACC-DKI mice were subjected to ex vivo isoproterenol perfusions with elevated afterload pressures to mimic higher workload conditions. This protocol increased workload in hearts from both ACC-DKI and controls by ~200% (Fig. 4A), which also increased FAO rates from baseline rates (Fig. 4B-dotted line) in hearts from both genotypes. Of importance, there was no significant difference between the WT and ACC-DKI mice with regard to heart rate and cardiac power at the elevated workload (Fig. 4C and D). Similarly, in vivo dobutamine stress echocardiography also demonstrated no differences between ACC-DKI mice and controls in terms of heart rate (Fig. 4E), ejection fraction (Fig. 4F) or isovolumic relaxation time (Fig. 4G), or any other echocardiographic parameters (Table 3). Together, these data support the concept that hearts from ACC-DKI mice are not energetically or functionally compromised at normal or elevated workloads.

Ischemia-induced activation of AMPK does not alter myocardial fatty acid oxidation rates in hearts of ACC-DKI mice.

Since ischemia-induced AMPK activation is thought to contribute to FAO dominating as the major source of ATP in the heart during reperfusion31, we tested whether preventing the AMPK-dependent inactivation of ACC would limit FAO following ischemia. To do this, we subjected hearts from WT and ACC-DKI mice to 18 minutes of ischemia, ex vivo. Following ischemia, hearts were reperfused to measure FAO rates. As expected, hearts from both genotypes demonstrated similar and significant ischemia-induced AMPK activation (Fig. 5A). This similar degree of AMPK activation also indicates that hearts from both genotypes of mice had equivalent levels of metabolic stress during the ischemic period. Consistent with no change in FAO rates in hearts from ACC-DKI mice compared to WT at baseline, preventing AMPK-mediated ACC inactivation did not affect FAO rates following ischemia in hearts from ACC-DKI mice (Fig. 5B). Interestingly, although we observed no changes in myocardial FAO rates in either genotypes, recovery of cardiac function following ischemia was significantly improved in ACC-DKI mice compared to controls (Fig. 5C and Table 2), suggesting a protection from ischemia-induced functional impairment in hearts from ACC-DKI mice.

DISCUSSION

To test the importance of the AMPK-dependent inhibitory phosphorylation of ACC in controlling cardiac FAO and function, we generated ACC-DKI mice and measured their cardiac performance at rest and during a variety of metabolic stresses. We show that in the absence of AMPK-mediated inactivation
of ACC, ACC-DKI hearts had a ~2-fold increase in myocardial malonyl CoA content compared to hearts from WT mice. These findings demonstrate that the AMPK-ACC signaling axis is essential for controlling malonyl CoA content in the heart, and that under normal physiological conditions, malonyl-CoA decarboxylase is unable to fully compensate to maintain malonyl-CoA content. Surprisingly, despite strong evidence indicating an important role for malonyl-CoA in the control of FAO, we show that hearts from ACC-DKI mice have normal rates of FAO and unaltered cardiac structure and function compared to controls. Thus, even though it is firmly established that ACC activity and malonyl CoA levels are key regulators of myocardial substrate utilization, our findings show that the ability of AMPK to inactivate ACC is not essential for maintaining FAO or cardiac function.

Since we show that AMPK-dependent inactivation of ACC is not essential for the control of myocardial FAO and subsequent cardiac function, we predict that additional mechanisms exist that allow for the maintenance of myocardial FAO rates independent from the AMPK-ACC-malonyl CoA signaling axis. We also suggest that these additional mechanisms are enhanced in the absence of AMPK-dependent inactivation of ACC and thus allow for easier identification. Consistent with this, we discovered that the protein expression of UCP3 is significantly higher in hearts of ACC-DKI mice compared to WT mice. Since UCP3 has been previously shown to increase FAO, a 3.6-fold compensatory increase in UCP3 expression may contribute to maintained FAO rates in hearts from the ACC-DKI mice. In agreement with UCP3 playing a role in hearts from ACC-DKI mice, higher levels of UCP3 protein expression have previously been shown to protect the heart from I/R injury as a result of preconditioning. Although we did not observe any differences in myocardial FAO rates following ischemia between genotypes, hearts from ACC-DKI mice demonstrated improved functional recovery following ischemia compared to controls. While the reason(s) for this unexpected improvement in functional recovery post-ischemia in the hearts from ACC-DKI needs further investigation, we speculate that higher expression of UCP3 in hearts from ACC-DKI mice may confer protection from ischemic injury as previously shown.

In summary, the data presented herein show for the first time that the AMPK-dependent inhibitory phosphorylation of ACC does not have an obligate role in the regulation of FAO in the healthy or stressed heart. Our findings raise the possibility that there are additional layers of FAO regulation, including up-regulation of UCP3, which are enhanced with the disruption of AMPK-dependent inhibitory phosphorylation of ACC. Our data suggest that while an intact AMPK-ACC signaling axis may be sufficient for the control of cardiac FAO, this pathway is not essential. These findings not only have broad implications about the fundamental mechanism involved in the regulation of FAO in the heart but may also influence how we interpret how/if AMPK activation controls myocardial FAO rates during pathological states or whether AMPK activation is beneficial or detrimental to the ischemic heart.

Limitations.

One important limitation of this study is that we cannot eliminate possible compensatory developmental changes resulting from the chronic ablation of AMPK signaling to ACC1 and ACC2 that maintain myocardial FAO. Thus, while AMPK-ACC signaling is not essential for regulating myocardial FAO, it may nevertheless be sufficient when present.
SOURCES OF FUNDING
This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to J.R.B.D and G.R.S. BEK is supported by the Australian NHMRC, ARC and in part by the Victorian Government Operational Infrastructure Support Program. B.Z. is supported by a postdoctoral fellowship from CIHR and a clinician fellowship from Alberta Innovates Health Solutions (AiHS). J.N. is supported by doctoral studentships from the Mazankowski Alberta Heart Institute, AiHS, and the Canadian Diabetes Association (CDA). T.P. is supported by a postdoctoral fellowship from AiHS. P.C.K. is supported by postdoctoral fellowships from the Heart and Stroke Foundation of Canada, the CDA, and the AiHS.

DISCLOSURES
None.

REFERENCES:

DOI: 10.1161/CIRCRESAHA.115.30453


### Table 1. Echocardiographic Parameters From WT and ACC-DKI Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>ACC-DKI</th>
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<tbody>
<tr>
<td><strong>Global Performance</strong></td>
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<td></td>
</tr>
<tr>
<td>Heart Rate, bpm</td>
<td>482 ± 8</td>
<td>465 ± 18</td>
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<tr>
<td>Tei Index</td>
<td>0.73 ± 0.02</td>
<td>0.71 ± 0.03</td>
</tr>
<tr>
<td><strong>Systolic Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ejection Fraction, %</td>
<td>62.4 ± 2.0</td>
<td>61.2 ± 3.0</td>
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<tr>
<td>Fractional Shortening, %</td>
<td>33.2 ± 1.4</td>
<td>32.6 ± 2.1</td>
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<tr>
<td><strong>Diastolic Function</strong></td>
<td></td>
<td></td>
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<tr>
<td>Isovolumic Relaxation Time, ms</td>
<td>16.9 ± 0.5</td>
<td>18.0 ± 0.7</td>
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<tr>
<td>E/A ratio</td>
<td>1.05 ± 0.06</td>
<td>1.05 ± 0.08</td>
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<tr>
<td>Mitral Valve Deceleration Time, ms</td>
<td>16.4 ± 1.2</td>
<td>17.4 ± 1.1</td>
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<tr>
<td>E/E'</td>
<td>22.6 ± 1.9</td>
<td>25.7 ± 2.0</td>
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<tr>
<td><strong>Wall Measurements</strong></td>
<td></td>
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<tr>
<td>Interventricular Septal Thickness - Diastole, mm</td>
<td>0.75 ± 0.03</td>
<td>0.76 ± 0.02</td>
</tr>
<tr>
<td>Interventricular Septal Thickness - Systole, mm</td>
<td>1.11 ± 0.04</td>
<td>1.15 ± 0.04</td>
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<tr>
<td>Left Ventricular Internal Diameter - Diastole, mm</td>
<td>3.87 ± 0.10</td>
<td>3.92 ± 0.06</td>
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<tr>
<td>Left Ventricular Internal Diameter - Systole, mm</td>
<td>2.58 ± 0.10</td>
<td>2.65 ± 0.11</td>
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<tr>
<td>Left Ventricular Posterior Wall - Diastole, mm</td>
<td>0.75 ± 0.02</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>Left Ventricular Posterior Wall - Systole, mm</td>
<td>1.11 ± 0.04</td>
<td>1.18 ± 0.05</td>
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Data are mean ± SEM of n = 7-8.
Table 2. Functional Parameters of *Ex vivo* Working Hearts from WT and ACC-DKI Mice Prior to and Following Ischemia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Aerobic</th>
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<th>Reperfusion</th>
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<td></td>
<td>WT</td>
<td>ACC-DKI</td>
<td>WT</td>
<td>ACC-DKI</td>
</tr>
<tr>
<td>Heart Rate, bpm</td>
<td>293.3 ± 6.77</td>
<td>313.4 ± 8.02</td>
<td>181.3 ± 22.95</td>
<td>224.1 ± 13.87</td>
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<tr>
<td>Peak Systolic Pressure, mmHg</td>
<td>63.23 ± 1.45</td>
<td>63.81 ± 1.08</td>
<td>21.60 ± 4.82</td>
<td>40.54 ± 5.32 *</td>
</tr>
<tr>
<td>Diastolic Pressure, mmHg</td>
<td>47.87 ± 1.19</td>
<td>48.54 ± 1.24</td>
<td>18.63 ± 4.13</td>
<td>32.46 ± 3.94 *</td>
</tr>
<tr>
<td>Developed Pressure, mmHg</td>
<td>15.38 ± 1.43</td>
<td>15.13 ± 0.91</td>
<td>3.00 ± 0.98</td>
<td>8.08 ± 1.77 *</td>
</tr>
<tr>
<td>Cardiac Output, ml/min</td>
<td>8.36 ± 0.49</td>
<td>9.01 ± 0.48</td>
<td>1.71 ± 0.55</td>
<td>3.39 ± 0.90</td>
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<td>Coronary Flow Rate, ml/min</td>
<td>2.55 ± 0.13</td>
<td>2.59 ± 0.11</td>
<td>1.37 ± 0.24</td>
<td>1.79 ± 0.31</td>
</tr>
<tr>
<td>Cardiac Power, mWatt</td>
<td>0.97 ± 0.07</td>
<td>1.05 ± 0.07</td>
<td>0.08 ± 0.06</td>
<td>0.33 ± 0.11 †</td>
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<tr>
<td>Cardiac Work, Joule/min</td>
<td>0.058 ± 0.004</td>
<td>0.063 ± 0.004</td>
<td>0.005 ± 0.003</td>
<td>0.020 ± 0.007 †</td>
</tr>
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Data are mean ± SEM of n = 10 - 16, *p* < 0.05 compared to WT mice within the same perfusion period, †p < 0.08 compared to WT mice within the same perfusion period.
### Table 3. Dobutamine Stress Echocardiographic Parameters From WT and ACC-DKI Mice

<table>
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<th>Parameters</th>
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<th>ACC-DKI</th>
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<td><strong>Global Performance</strong></td>
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<tr>
<td>Heart Rate, bpm</td>
<td>524 ± 7</td>
<td>543 ± 10</td>
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<tr>
<td>Tei Index</td>
<td>0.69 ± 0.03</td>
<td>0.69 ± 0.03</td>
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<td><strong>Systolic Function</strong></td>
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<tr>
<td>Ejection Fraction, %</td>
<td>79.9 ± 3.0</td>
<td>81.8 ± 3.7</td>
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<tr>
<td>Fractional Shortening, %</td>
<td>48.8 ± 3.7</td>
<td>51.0 ± 4.0</td>
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<tr>
<td><strong>Diastolic Function</strong></td>
<td></td>
<td></td>
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<tr>
<td>Isovolumic Relaxation Time, ms</td>
<td>15.6 ± 0.8</td>
<td>15.2 ± 0.7</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.08 ± 0.08</td>
<td>1.02 ± 0.04</td>
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<tr>
<td>Mitral Valve Deceleration Time, ms</td>
<td>13.1 ± 1.6</td>
<td>16.9 ± 2.1</td>
</tr>
<tr>
<td>E/E’</td>
<td>19.8 ± 1.2</td>
<td>23.1 ± 1.3</td>
</tr>
<tr>
<td><strong>Wall Measurements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interventricular Septal Thickness - Diastole, mm</td>
<td>0.91 ± 0.07</td>
<td>0.90 ± 0.04</td>
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<tr>
<td>Interventricular Septal Thickness - Systole, mm</td>
<td>1.43 ± 0.08</td>
<td>1.51 ± 0.10</td>
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<tr>
<td>Left Ventricular Internal Diameter - Diastole, mm</td>
<td>3.56 ± 0.08</td>
<td>3.54 ± 0.05</td>
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<tr>
<td>Left Ventricular Internal Diameter - Systole, mm</td>
<td>1.83 ± 0.2</td>
<td>1.74 ± 0.16</td>
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<tr>
<td>Left Ventricular Posterior Wall - Diastole, mm</td>
<td>0.83 ± 0.05</td>
<td>0.87 ± 0.04</td>
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<tr>
<td>Left Ventricular Posterior Wall - Systole, mm</td>
<td>1.42 ± 0.06</td>
<td>1.53 ± 0.08</td>
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Data are mean ± SEM of n= 7-8.
FIGURE LEGENDS

Figure 1. Characterization of ACC-DKI hearts. A. Generation of ACC-DKI mice. DNA sequences encoding for amino acids Ser79 and Ser212 were mutated to alanines (mutations in red) and ACC1 (ACC1 DS79A) and ACC2 (ACC2 DS212A) knock-in mice were generated. These two strains were crossed to produce ACC-DKI mice. B. Immunoblot analysis of P-ACC and total ACC protein expression. Levels of P-ACC were quantified by densitometry and normalized against total ACC (n = 3, *p < 0.05). C. Immunoblot analysis of P-AMPK Thr172 and total AMPK protein expression. Levels of P-AMPK were quantified by densitometry and normalized against total AMPK (n = 3).

Figure 2. Structural, functional, and metabolic characterization of ACC-DKI hearts at baseline. A. Representative whole heart sections stained with Masson trichrome for visualizing structural morphology (n = 4). B. Ventricular weight/tibial length, mg/mm (n = 4). C. Ejection fraction, % (n = 15). D. Isovolumic relaxation time, ms (n = 15). E. Myocardial Malonyl CoA content from frozen ex vivo perfused hearts, nmol/g wet wt (n = 4 – 5, *p < 0.05). F. Ex vivo palmitate oxidation rate, nmol/g dry wt/min (n = 12 – 16).

Figure 3. Expression of proteins involved in cardiac substrate metabolism. A. Immunoblot analysis of ATGL, CD36, FATP1, and PPARα proteins in WT and ACC-DKI hearts, levels of the proteins were quantified by densitometry and normalized against Actin (n = 3). B. Immunoblot analysis of P-Akt Ser473, P-Akt Thr308, P-p38 Thr180, and P-PKA Thr197 proteins in WT and ACC-DKI hearts, levels of the phospho-proteins were normalized against the corresponding total protein (n = 3). C. Immunoblot analysis of UCP3 in WT and ACC-DKI hearts, levels of the proteins were quantified by densitometry and normalized against Actin (n = 3, *p < 0.05).

Figure 4. Ex vivo energy metabolism and in vivo myocardial function during high workload. Ex vivo isoproterenol working heart perfusions - A. Rate pressure product, mmHg/min (n = 5 – 6). B. Palmitate oxidation rates, nmol/g dry wt/min (n = 4 – 5). C. Heart rate, bpm (n = 4 – 5). D. Cardiac power, mWatt (n = 4 – 6). In vivo dobutamine infusion - E. Heart rate, bpm (n = 4 – 5). F. Ejection fraction, % (n = 4 – 5) and G. Isovolumic relaxation time, ms (n = 4 – 5). Dotted lines represent baseline measurements.

Figure 5. Ex Vivo myocardial energy metabolism and function during ischemia and reperfusion. A. Immunoblot analysis of P-AMPK Thr172 and total AMPK protein expression. Levels of P-AMPK were quantified by densitometry and normalized against total AMPK (n = 3, *p < 0.05). B. Palmitate oxidation rates, nmol/g dry wt/min (n = 4 – 5). C. Rate pressure product, mmHg/min (n = 4 – 5, *p < 0.05).
Novelty and Significance

What Is Known?

- Adenosine monophosphate-activated protein kinase (AMPK) is a major regulator of cardiac energy metabolism and function.

- AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC) to increase rates of myocardial fatty acid oxidation (FAO) and promote ATP production.

- Alanine knock-in mutations in both ACC1 and ACC2 (ACC-DKI) render ACC resistant to inhibitory phosphorylation by AMPK, which inhibits FAO in organs other than the heart.

What new information does this article contribute?

- In contrast to other organs, FAO is not impaired in the healthy or stressed heart of ACC-DKI mice.

- ACC-DKI mutant mice have normal cardiac function at baseline and during elevated workloads.

- AMPK-dependent inactivation of ACC is not essential for the maintenance of myocardial FAO and subsequent cardiac function since other molecular pathways can compensate for the loss of AMPK-ACC signaling.

The ability of AMPK to phosphorylate and inhibit ACC leads to accelerated rates of FAO. However, whether or not the AMPK-ACC signaling axis plays an obligate role in the maintenance of myocardial FAO rates has not been firmly established. In the present study, we used mutant mice with alanine knock-in mutations in both ACC1 (at Ser79) and ACC2 (at Ser212) thereby rendering ACC resistant to inhibitory phosphorylation by AMPK yet leaving AMPK and ACC activity intact. Using these ACC double knock-in (ACC-DKI) mice, we show that deletion of the AMPK-ACC signaling axis does not lead to alterations in cardiac FAO rates and thus challenges the existing dogma that the AMPK-ACC signaling axis plays an obligate role in the maintenance of myocardial FAO rates. Together, our findings have broad implications about the fundamental mechanisms involved in the regulation of FAO in the heart and suggest that other mechanisms can compensate for the loss of AMPK-ACC signaling to maintain cardiac FAO.
Fig. 1

(A) Diagram showing the sequence variations and genetic implications of ACC1 and ACC2 mutations.

(B) Western blot analysis of P-ACC and Total ACC in WT and ACC-DKI conditions.

(C) Western blot analysis of P-AMPK and Total AMPK in WT and ACC-DKI conditions.
Fig. 2

(A) WT and ACC-DKI hearts

(B) Ventricular Weight/Trabecula Length (mg/mm)

(C) Ejection Fraction [%]

(D) Isovolumic Relaxation Time [ms]

(E) Malonyl CoA content [nmol/g wet wt]

(F) Palmitate Oxidation [nmol/g dry wt]
Fig. 3

(A) Graph showing arbitrary densitometric units for ATGL, CD36, FATP1, and PPARγ.

(B) Graph showing arbitrary densitometric units for P-Akt Ser473, P-Akt Thr308, P-p38 Thr180, and P-PKA Thr197.

(C) Western blot images of UCP3 and Actin for WT and ACC-DKI conditions, with a quantified bar graph comparing UCP3/Actin levels.
Fig. 4

(A) Graph showing HR x PSP (10^3) (mmHg/min) vs Time [min] with data points for WT and ACC-DKI.

(B) Bar graph comparing Palmitate Oxidation [nmol/g dry wt/min] for WT and ACC-DKI.

(C) Bar graph showing Heart Rate [bpm] for WT and ACC-DKI.

(D) Bar graph comparing Cardiac Power, mWatt for WT and ACC-DKI.

(E) Bar graph showing Heart Rate [bpm] for WT and ACC-DKI.

(F) Bar graph comparing Ejection Fraction [%] for WT and ACC-DKI.

(G) Bar graph showing Isovolumic Relaxation Time [ms] for WT and ACC-DKI.
Fig. 5

(A) P-AMPK and Total AMPK expression in WT and ACC-DKI mice under aerobic and reperfusion conditions.

(B) Fatidate oxidation levels in WT and ACC-DKI mice.

(C) Heart rate x PSP (mmHg/min) over time following ischemia in WT and ACC-DKI mice.
AMPK-Dependent Inhibitory Phosphorylation of ACC Is Not Essential for Maintaining Myocardial Fatty Acid Oxidation
Beshay N Zordoky, Jeevan Nagendran, Thomas Pulinilkunnil, Petra C Kienesberger, Grant Masson, Terri J Waller, Bruce E Kemp, Greg Steinberg and Jason R Dyck

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Supplemental Material

METHODS:

Animals

ACC-DKI mice that express the two mutant isoforms of ACC that are resistant to inhibitory phosphorylation by AMPK were described previously\(^1\). For all experiments, mice of 20-33 weeks old were studied, littermate wild-type (WT) mice were used as controls and since there were no sex differences in the parameters measured, both male and female mice were used. The University of Alberta Institutional Animal Care and Use Committee approved all protocols involving mice.

Echocardiography and Dobutamine Stress Echocardiography

Mice were mildly anesthetized using isoflurane, and transthoracic echocardiography was performed using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada) as previously described\(^2,3\). To assess in vivo heart function under high workload conditions, dobutamine was administered at a dose of 4 mg/kg via intraperitoneal injections during echocardiography. Ten minutes after dobutamine injection, in vivo function was assessed.

Heart Perfusions

Hearts were perfused in the working heart mode at 11.5 mm Hg preload and 50 mm Hg afterload with Krebs-Henseleit buffer containing 1.2 mmol/L palmitate prebound to 3% delipidated bovine serum albumin (BSA), 5 mmol/L glucose, and 50 µU/mL insulin. To assess ex vivo heart function under high workload conditions, hearts were perfused for an initial period of 30 min at normal workload followed by perfusion at 80 mm Hg afterload and additional 300 nmol/L isoproterenol in the perfusion buffer for a duration of 30 min\(^2\). For ischemia-reperfusion studies, hearts were aerobically perfused for 30 minutes, or aerobically perfused for 30 minutes followed by 18 minutes of global no flow ischemia and 40 minutes of reperfusion. At the end of the perfusion, hearts were immediately frozen in liquid N\(_2\) with a Wollenberger clamp and stored at -80°C as described previously\(^4\). For metabolic measurements, a tracer of [9,10-3H]palmitate was used to determine FAO as previously described\(^4\).

Tissue Homogenization and Immunoblot Analysis

Frozen hearts were ground using mortar and pestle, and tissue powder was homogenized in ice-cold lysis buffer containing 20 mmol/liter Tris-HCl (pH 7.4), 5 mmol/liter EDTA, 10 mmol/liter Na\(_4\)P\(_2\)O\(_7\), 100 mmol/liter NaF, 1% Nonidet P-40, 2 mmol/liter Na\(_3\)VO\(_4\), protease inhibitor (product number P8340, 10µl/ml; Sigma, St. Louis, MO), and phosphatase inhibitor (product number 524628, 20 µg/ml; Calbiochem, EMD Chemicals, Gibbstown, NJ) unless otherwise stated. Homogenates were centrifuged at 1,200 x g for 20 min at 4°C, and the supernatants were transferred to fresh tubes. Protein concentration in lysates was determined using the bicinchoninic acid (BCA) protein assay kit (product number 23255; Pierce, Thermo Fisher Scientific, Rockford, IL), and serum albumin was employed as the standard (product number 23210; Pierce). Lysates were aliquoted and stored at -80°C until further usage for immunoblot analysis. Tissue lysates were resolved by SDS-PAGE, and proteins were transferred onto a nitrocellulose membrane. Blotted proteins were reversibly visualized using MemCode stain (Pierce) and identified using the primary antibodies: anti-ACC (EMD Millipore, Billerica, MA), anti-AMPK\(\alpha_2\), anti-fatty acid transport protein 1 (FATP1), anti-peroxisome proliferator-activated receptor alpha (PPAR\(\alpha\)), anti-actin (Santa Cruz Biotechnology, Dallas, TX), anti-cluster of differentiation 36 (CD36) (generated in-house), anti-phospho ACC Ser79, anti-phospho AMPK Thr172, anti-adipose triglyceride lipase (ATGL), anti-phospho Akt Ser473,
anti-phospho Akt Thr308, anti-Akt, ant-phospho p38 Thr108, anti-p38, anti-phospho protein kinase A (PKA) Thr197, anti-PKA (Cell Signaling Technology, Danvers, MA) antibodies. Immunoblots were developed using the Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Waltham, MA). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). All densitometric data were corrected against total protein loading visualized via Memcode (Pierce) staining.

**Malonyl CoA Measurements**

Following heart perfusions, malonyl CoA was extracted from 20 to 30 mg of frozen ventricular tissue by homogenization in ice-cold 6% (vol/vol) perchloric acid-1 mmol/liter dithiothreitol-0.5 mmol/liter EGTA. Homogenates were spun at 12,000 x g for 5 min at 4°C. Half of the homogenate was then used for quantification of malonyl CoA content by Ultra-high performance liquid chromatography (UPLC)\(^1\)\(^5\).

**Statistical Analysis**

Results are expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism software. Comparisons between two groups were made by unpaired two-tailed Student’s t-test. Comparisons between two or more groups were made by one way ANOVA and comparisons involving multiple time points are by two way ANOVA respectively. P-values of less than 0.05 were considered statistically significant.

**References**