AMPK-Dependent Inhibitory Phosphorylation of ACC Is Not Essential for Maintaining Myocardial Fatty Acid Oxidation

Beshay N.M. Zordoky,* Jeevan Nagendran,* Thomas Pulinilkunnil,* Petra C. Kienesberger, Grant Masson, Terri J. Waller, Bruce E. Kemp, Gregory R. Steinberg, Jason R.B. Dyck

Rationale: The energy sensor AMP-activated protein kinases (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 preventing 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 with wild-type mice. However, cardiac FAO rates were comparable between wild-type and ACC-DKI mice at baseline, during elevated workloads, and after 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 AMPK-dependent metabolic adaptations. (Circ Res. 2014;115:518-524.)

Key Words: acetyl-CoA carboxylase ■ AMP-activated protein kinase

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

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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 phosphorylation. 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 liver. However, the effect of preventing AMPK-mediated phosphorylation of ACC in the heart has not been investigated. Herein, we show that hearts from ACC-DKI mice have increased malonyl-CoA levels compared with wild-type (WT) mice, but their FAO rates...
are comparable with WT either at baseline or during elevated workloads and after myocardial ischemia; conditions known to result in AMPK-independent12,13 and AMPK-dependent metabolic adaptations, 14,15 respectively. Together, these findings show that during work-induced metabolic adaptations and 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 2 mutant isoforms of ACC that are resistant to inhibitory phosphorylation by AMPK were described previously.11 For all experiments, mice of 20 to 33 weeks old were studied; littermate WT mice were used as controls and because 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 is available in the online-only Data Supplement.

**Baseline Echocardiography and Dobutamine Stress Echocardiography**

In vivo cardiac function was assessed using transthoracic echocardiography at baseline and at 10 minutes after dobutamine injection as previously described.16,17

**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 previously.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, as described previously.18

**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 blotted and 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**

After heart perfusions, malonyl-CoA was extracted from 20 to 30 mg of frozen ventricular tissue and quantified by ultra-high performance liquid chromatography.11,19

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>acetyl coenzyme A carboxylase</td>
</tr>
<tr>
<td>ACC-DKI</td>
<td>ACC-double knock-in</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>FAO</td>
<td>fatty acid oxidation</td>
</tr>
<tr>
<td>UCP3</td>
<td>uncoupling protein-3</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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</table>

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Malonyl-CoA Measurements

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**Figure 1.** Characterization of acetyl coenzyme A carboxylase double knock-in (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 ΔS79A) and ACC2 (ACC2 ΔS212A) knock-in mice were generated. These 2 strains were crossed to produce ACC-DKI mice. B, Immunoblot analysis of phosphorylated-ACC (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 phosphorylated AMP-activated protein kinase (P-AMPK) Thr172 and total AMPK protein expression. Levels of P-AMPK were quantified by densitometry and normalized against total AMPK (n=3). WT indicates wild-type.

**Figure 2.** Structural, functional, and metabolic characterization of acetyl coenzyme A carboxylase double knock-in (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). WT indicates wild-type.
Loss of AMPK-Mediated ACC Phosphorylation in Hearts of ACC-DKI Mice Does Not Alter Myocardial FAO Rates

As expected with the loss of AMPK-mediated inhibitory phosphorylation of ACC, malonyl-CoA levels were elevated in ACC-DKI mice compared with controls, both in the ex vivo perfused hearts (Figure 2E) and in the 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,11 ex vivo perfused hearts from these mice show no differences in myocardial FAO rates (Figure 2F) compared with hearts from WT mice. This lack of effect on myocardial FAO rates is consistent with the normal function of these hearts during ex vivo perfusion (Table 2; first two columns).

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, cluster differentiation 36, fatty acid transport protein 1, or peroxisome proliferator-activated receptor α20–23 (Figure 3A).

Table 2. Functional Parameters of Ex Vivo Working Hearts From WT and ACC-DKI Mice Before and After Ischemia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>ACC-DKI</th>
<th>WT</th>
<th>ACC-DKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>293±3.6</td>
<td>313±4.8</td>
<td>181±2.9</td>
<td>224±1.3</td>
</tr>
<tr>
<td>Peak systolic pressure, mm Hg</td>
<td>63.2±1.5</td>
<td>63.8±1.0</td>
<td>21.6±4.2</td>
<td>40.5±5.3</td>
</tr>
<tr>
<td>Diastolic pressure, mm Hg</td>
<td>48.7±1.2</td>
<td>48.5±1.2</td>
<td>18.6±4.3</td>
<td>32.4±3.9</td>
</tr>
<tr>
<td>Developed pressure, mm Hg</td>
<td>15.3±0.9</td>
<td>15.1±0.9</td>
<td>3.0±0.9</td>
<td>8.0±1.7</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>8.36±0.4</td>
<td>9.01±0.4</td>
<td>1.71±0.5</td>
<td>3.39±0.9</td>
</tr>
<tr>
<td>Coronary flow rate, mL/min</td>
<td>2.55±0.1</td>
<td>2.59±0.1</td>
<td>1.37±0.2</td>
<td>1.79±0.3</td>
</tr>
<tr>
<td>Cardiac power, mW</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>
</tr>
<tr>
<td>Cardiac work, J/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>
</tbody>
</table>

Data are mean±SEM of n=10 to 16. ACC-DKI indicates acetyl coenzyme A carboxylase double knock-in; and WT, wild-type.

*P<0.05 compared with WT mice within the same perfusion period.
†P<0.08 compared with WT mice within the same perfusion period.
which are proteins involved in glucose metabolism, as well as in the regulation of FAO. 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 with the hearts of WT (Figure 3C). This is particularly important because UCP3 has been previously shown to increase FAO rates and thus may be responsible for normal FAO rates observed in hearts from ACC-DKI mice despite a disrupted AMPK-ACC signaling axis.

**Figure 3. Expression of proteins involved in cardiac substrate metabolism.** A, Immunoblot analysis of adipose triglyceride lipase (ATGL), cluster of differentiation 36 (CD36), fatty acid transport protein 1 (FATP1), and peroxisome proliferator-activated receptor α (PPARα) proteins in wild-type (WT) and acetyl coenzyme A carboxylase double knock-in (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 phosphorylated protein kinase A (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 uncoupling protein-3 (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, heart rate (HR) x peak systolic pressure (PSP), 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, mW (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.

**Loss of AMPK-Mediated ACC Phosphorylation in Hearts of ACC-DKI Mice Does Not Alter Myocardial FAO or Function at Elevated Workloads**

Because 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% (Figure 4A), which also increased FAO rates from baseline rates (Figure 4B, dotted line) in hearts from both genotypes. Of importance, there was no significant difference between the WT and the ACC-DKI mice with regard to heart rate and cardiac power at the elevated workload (Figure 4C and 4D). Similarly, in vivo dobutamine stress echocardiography also demonstrated no differences between ACC-DKI mice and controls in terms...
of heart rate (Figure 4E), ejection fraction (Figure 4F) or isovolumic relaxation time (Figure 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 FAO Rates in Hearts of ACC-DKI Mice

Because ischemia-induced AMPK activation is thought to contribute to FAO dominating as the major source of ATP in the heart during reperfusion, we tested whether preventing the AMPK-dependent inactivation of ACC would limit FAO after ischemia. To do this, we subjected hearts from WT and ACC-DKI mice to 18 minutes of ischemia, ex vivo. After ischemia, hearts were reperfused to measure FAO rates. As expected, hearts from both genotypes demonstrated similar and significant ischemia-induced AMPK activation (Figure 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 with hearts from WT at baseline, preventing AMPK-mediated ACC inactivation did not affect FAO rates after ischemia in hearts from ACC-DKI mice (Figure 5B). Interestingly, although we observed no changes in myocardial FAO rates in either genotypes, recovery of cardiac function after ischemia was significantly improved in ACC-DKI mice compared with controls (Figure 5C; 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 with 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

Table 3. Dobutamine Stress Echocardiographic Parameters From WT and ACC-DKI Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>ACC-DKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global performance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>524±7</td>
<td>543±10</td>
</tr>
<tr>
<td>Tei index</td>
<td>0.69±0.03</td>
<td>0.69±0.03</td>
</tr>
<tr>
<td>Systolic function, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>79.9±3.0</td>
<td>81.8±3.7</td>
</tr>
<tr>
<td>Fractional shortening</td>
<td>48.8±3.7</td>
<td>51.0±4.0</td>
</tr>
<tr>
<td>Diastolic function</td>
<td></td>
<td></td>
</tr>
<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>
</tr>
<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>Wall measurements, mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interventricular septal thickness: diastole</td>
<td>0.91±0.07</td>
<td>0.90±0.04</td>
</tr>
<tr>
<td>Interventricular septal thickness: systole</td>
<td>1.43±0.08</td>
<td>1.51±0.10</td>
</tr>
<tr>
<td>Left ventricular internal diameter: diastole</td>
<td>3.56±0.08</td>
<td>3.54±0.05</td>
</tr>
<tr>
<td>Left ventricular internal diameter: systole</td>
<td>1.83±0.2</td>
<td>1.74±0.16</td>
</tr>
<tr>
<td>Left ventricular posterior wall: systole</td>
<td>0.83±0.05</td>
<td>0.87±0.04</td>
</tr>
<tr>
<td>Left ventricular posterior wall: systole</td>
<td>1.42±0.06</td>
<td>1.53±0.08</td>
</tr>
</tbody>
</table>

Data are mean±SEM of n=7 to 8. ACC-DKI indicates acetyl coenzyme A carboxylase double knock-in; and WT, wild-type.
mice have normal rates of FAO and unaltered cardiac structure and function compared with controls. Thus, even though it is firmly established that ACC activity and malonyl-CoA levels are key regulators of myocardial substrate use,

our findings show that the ability of AMPK to inactivate ACC is not essential for maintaining FAO or cardiac function.

Because 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 with hearts of WT mice. Because UCP3 has been previously shown to increase FAO,

a 3.6-fold compensatory increase in UCP3 expression may contribute to maintain 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 ischemia-reperfusion injury as previously shown.

Despite this, we discovered that the protein expression of UCP3 in hearts from the chronic ablation of AMPK signaling to ACC1 and ACC2 that maintain myocardial FAO. Thus, although 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. Dyck and G.R. Steinberg. G.R. Steinberg is supported by the Canadian Diabetes Association (CDA) and is a Canada Research Chair in Metabolism and Obesity. B.E. Kemp is supported by the Australian National Health and Medical Research Council, Australian Research Council and, in part, by the Victorian Government Operational Infrastructure Support Program. B.N.M. Zordoky is supported by a postdoctoral fellowship from CIHR and a clinician fellowship from Alberta Innovates Health Solutions (AIHS). J. Nagendran is supported by doctoral studentships from the Mazankowski Alberta Heart Institute, AIHS, and the CDA. T. Pulinkulkunnal is supported by a postdoctoral fellowship from AIHS. P.C. Kienesberger is supported by postdoctoral fellowships from the Heart and Stroke Foundation of Canada, the CDA, and the AIHS.

Disclosures

None.

References


Novelty and Significance

What Is Known?

- AMP-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 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-double knock-in mice.
- ACC-double knock-in 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 because 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 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 expression intact. Using these ACC-double knock-in mice, we show that disruption 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.
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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 Perfusion
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-\(^3\)H]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\(_2\)P\(_2\)O\(_7\), 100 mmol/liter NaF, 1% Nonidet P-40, 2 mmol/liter Na\(_3\)VO\(_4\), protease inhibitor (product number P8340, 10 µl/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**