Fatty Acids Attenuate Insulin Regulation of 5’-AMP–Activated Protein Kinase and Insulin Cardioprotection After Ischemia

Clifford D.L. Folmes, Alexander S. Clanachan, Gary D. Lopaschuk

Abstract—The cardioprotective effect of insulin during ischemia–reperfusion has been associated with stimulation of glucose uptake and glycolysis. Although fatty acids and 5’-AMP activated protein kinase (AMPK) are regulators of glucose metabolism, it is unknown what effect insulin has on postischemic function and AMPK activity in the presence of high levels of fatty acid. Isolated ejection mouse hearts were perfused with Krebs–Henseleit solution containing 5 mmol·L\(^{-1}\) glucose and 0, 0.2, or 1.2 mmol·L\(^{-1}\) palmitate, with or without 100 μU/mL insulin. During aerobic perfusion in the absence of palmitate, insulin stimulated glycolysis by 73% and glucose oxidation by 54%, while inhibiting AMPK activity by 43%. In the presence of 0.2 or 1.2 mmol·L\(^{-1}\) palmitate, insulin stimulated glycolysis by 111% and 105% and glucose oxidation by 72% and 274% but no longer inhibited AMPK activity. During reperfusion of hearts in the absence of palmitate, insulin increased recovery of cardiac power by 47%. This was associated with a 97% increase in glycolysis and a 160% increase in glucose oxidation. However, in the presence of 1.2 mmol·L\(^{-1}\) palmitate, insulin now decreased recovery of cardiac power by 42%. During reperfusion, glucose oxidation was inhibited by high fat, but insulin-stimulated glycolysis remained high, resulting in increased proton production. In the absence of fatty acids, insulin blunted the ischemia-induced activation of AMPK, but this effect was lost in the presence of fatty acids. We demonstrate that the cardioprotective effect of insulin and its ability to inhibit AMPK activity are lost in the presence of high concentrations of fatty acids. (Circ Res. 2006;99:61-68.)

Key Words: fatty acid oxidation ■ glucose oxidation ■ glycolysis ■ proton production ■ ischemia–reperfusion
an acceleration of fatty acid oxidation in the heart, which, in turn, leads to a decrease in glucose oxidation.\textsuperscript{14} Of potential importance is that we and others have demonstrated that insulin can inhibit the activation of AMPK during both aerobic perfusion and ischemia in the presence or absence of normal concentrations of fatty acids (0.4 mmol·L\textsuperscript{-1} palmitate bound to 3% BSA or 0 mmol·L\textsuperscript{-1} palmitate, respectively); however, recovery of postischemic function was not assessed in these studies. Therefore, insulin may have a salutary effect in ischemia by indirectly inhibiting fatty acid oxidation.

Under normal aerobic conditions, the heart preferentially metabolizes fatty acids, which contribute between 60% and 80% of the energy requirements of the heart. However, during and following most clinically relevant forms of myocardial ischemia, the heart is exposed to high levels of circulating fatty acids.\textsuperscript{15} When hearts are reperfused following severe ischemia, fatty acid oxidation quickly recovers and predominates as the main source of mitochondrial oxidative metabolism.\textsuperscript{16–18} This is attributable both to the exposure of the heart to a high concentration of fatty acids as well as to subcellular changes in the control of fatty acid oxidation.\textsuperscript{14} During ischemia, anaerobic glycolysis increases and becomes an important source of ATP production that has a role in the maintenance of ion gradients in the cardiomyocyte.\textsuperscript{19} However, if the pyruvate from glycolysis is not subsequently oxidized, such as in the presence of high fatty acids, there is a production of both lactate and protons, the latter produced by the hydrolysis of glycolytically derived ATP. This proton production is a major contributor to the intracellular acidosis that is associated with ischemia.\textsuperscript{20} The acidosis can lead to a sequence of adverse events, including accelerated sarcoplasmic Na\textsuperscript{+}/H\textsuperscript{+} exchange leading to intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} overload,\textsuperscript{21,22} decreased cardiac pressure development,\textsuperscript{3} the initiation of cardiac arrhythmias,\textsuperscript{23} and decreased response of contractile proteins to Ca\textsuperscript{2+}.\textsuperscript{24} If the pyruvate from glycolysis is aerobically metabolized (ie, glucose oxidation), then lactate and protons are not produced.\textsuperscript{24,25}

Despite the importance of insulin, AMPK, and fatty acids in controlling glucose metabolism in the heart, little is known about their interaction during and following ischemia. Recently, Clark et al showed that fatty acids can attenuate the ability of insulin to inhibit AMPK activation.\textsuperscript{26} Because fatty acids are elevated in most clinical forms of myocardial ischemia, it is possible that high fatty acids overcome any beneficial effects of insulin during ischemia. Therefore, we investigated the effect of insulin on AMPK activation and myocardial metabolism in the presence of graded concentrations of fatty acids. In addition, we considered the implications of these observations on AMPK activation during ischemia and the effect on recovery of contractile function following ischemia.

### Materials and Methods

#### Animals

The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with Canadian Council of Animal Care guidelines.

#### Isolated Ejecting Mouse Heart Model

CD-1 mouse hearts (98 mice) were perfused in the working ejection mode as previously described.\textsuperscript{28} Briefly, mice were anesthetized with pentobarbital sodium (12 mg IP), and the hearts were subsequently excised and cannulated via the aorta and left atrium. After equilibration in the Langendorff mode, hearts were switched to the ejection mode and perfused with modified Krebs–Henseleit solution containing 118.5 mmol·L\textsuperscript{-1} NaCl, 25 mmol·L\textsuperscript{-1} NaHCO\textsubscript{3}, 4.7 mmol·L\textsuperscript{-1} KCl, 1.2 mmol·L\textsuperscript{-1} MgSO\textsubscript{4}, 1.2 mmol·L\textsuperscript{-1} KH\textsubscript{2}PO\textsubscript{4}, 2.5 mmol·L\textsuperscript{-1} CaCl\textsubscript{2}, 0.5 mmol·L\textsuperscript{-1} EDTA, 5 mmol·L\textsuperscript{-1} [5\textsuperscript{-14}C]glucose, and 3% fatty acid-free albumin. Hearts were perfused in the presence or absence of 100 µU/mL insulin, depending on the experimental protocol. The oxygenated Krebs–Henseleit solution contained either no fatty acids (no fat) or 0.2 (low fat) or 1.2 mmol·L\textsuperscript{-1} (high fat) palmitate. The palmitate was prebound to the albumin as described previously.\textsuperscript{26} Glycolysis and glucose oxidation were measured by quantitative collection of the H\textsubscript{2}O and 14CO\textsubscript{2}, respectively, derived from [5\textsuperscript{-14}C]glucose, as described previously (see the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org).\textsuperscript{24,29}

Spontaneously beating hearts were perfused at a constant left atrial preload pressure of 11.5 mm Hg and a constant aortic afterload pressure of 50 mm Hg for: (1) a 40-minute aerobic perfusion; (2) a 30-minute aerobic perfusion followed by 16 minutes (no fat) or 18 minutes (high fat) of global no-flow ischemia and 40 minutes of aerobic reperfusion; (3) a 30-minute aerobic control; and (4) a 30-minute aerobic perfusion followed by 16 minutes (no fat) or 18 minutes (high fat) of global no-flow ischemia, for an ischemia control (supplemental Figure I). At the end of the perfusion protocol, the hearts were quick frozen in liquid nitrogen with Wollenberger tongs and stored at −80°C.

#### Tissue Preparation for AMPK Activity Assay

Approximately 20 mg of frozen ventricular tissue was homogenized for 30 seconds using a Polytron Homogenizer in 80 µL of homogenization buffer containing 50 mmol·L\textsuperscript{-1} Tris-HCl (pH 8 at 4°C), 1 mmol·L\textsuperscript{-1} EDTA, 10% (wt/vol) glycerol, 0.02% (vol/vol) Brij-55, 1 mmol·L\textsuperscript{-1} dithiothreitol (DTT), protease inhibitors (Sigma), and phosphatase inhibitors (Sigma). The homogenates were centrifuged at 10 000 g for 20 minutes at 4°C, and the resultant supernatant was used to assay AMPK activity, as previously described, except the synthetic AMARA peptide was used as the substrate.\textsuperscript{11}

#### Statistical Analysis

All data are presented as the mean±SEM. The data were analyzed with the statistical program Instat 2.01 and GB-stat. Two-way repeated-measures ANOVA with a Bonferroni post hoc test was used to evaluate the statistical significance of differences among groups for cardiac power. One-way or 2-way ANOVA with a Bonferroni post hoc test was used to evaluate the statistical significance of differences among groups for the metabolic data. For the AMPK activity data, a t test was used to determine differences between the no-insulin and insulin groups at the 3 different time points (aerobic, ischemia, and reperfusion) and then a 2-way ANOVA to determine the difference between the time points. Values of P<0.05 were considered significant.

### Results

#### Baseline Aerobic Values in Forty-Minute Aerobic Hearts Perfused in the Absence of Fatty Acids

The addition of insulin to aerobically perfused mouse hearts in the absence of fatty acids caused a significant increase in cardiac power (71±4 to 87±6 mJ·min\textsuperscript{-1}, P<0.05, for the no-insulin and insulin groups, respectively). Heart rate, cardiac output, and coronary flow did not differ between these groups (data not shown). As expected, insulin also increased glucose metabolism in these hearts, resulting in a 73% increase in glycolysis (Figure 1B; P<0.05) and a 54%
increase in glucose oxidation (Figure 1C; \( P<0.05 \)). Insulin also resulted in a 43% decrease in AMPK activity (Figure 1A; \( P<0.05 \)).

Immunoblot analysis showed that insulin produces a 3-fold increase in phosphorylation of Akt at Ser473 (supplemental Figure IIA). Despite this activation of Akt, there was no downstream effect on phosphorylation of AMPK at Ser485/491 (supplemental Figure IIB).

**Baseline Aerobic Values in Forty-Minute Aerobic Hearts Perfused in the Presence of Fatty Acids**

The addition of insulin to perfused mouse hearts in the presence of either 0.2 or 1.2 mmol·L\(^{-1}\) palmitate did not significantly modify cardiac power or any of the other parameters of mechanical function (data not shown). Despite the lack of change in mechanical function, insulin resulted in a 111% increase in glycolysis in the low-fat group (Figure 2B; \( P<0.05 \)) and a 106% increase in glycolysis in the high-fat group (Figure 2B; \( P<0.05 \)). Insulin also increased glucose oxidation by 72% in the low-fat group (Figure 2B; \( P<0.05 \)) and by 273% in the high-fat group (Figure 2C; \( P<0.05 \)). However, in the presence of both low and high fat, insulin no longer inhibited AMPK activity (Figure 2A).

Immunoblot analysis also showed that in the presence of high fat, insulin produces a 2.5-fold increase in phosphorylation of Akt at Ser473 (supplemental Figure IA) and, as seen in the no fat hearts, there is no downstream effect on phosphorylation of AMPK at Ser485/491 (supplemental Figure IB).

**Effect of Insulin and Palmitate on Myocardial Metabolism and Functional Recovery During Reperfusion Following Global No-Flow Ischemia**

**Ischemia and Reperfusion in the Absence of Fatty Acids**

Insulin produced a small but insignificant increase in cardiac output and cardiac power and did not significantly change any other parameters of mechanical function of hearts per-
fused in the absence of fatty acids during aerobic perfusion (Figure 3 and supplemental Table I). Similar to the previous aerobically perfused group, insulin increased glycolysis by 73% (Figure 4A) and glucose oxidation by 45% during the initial aerobic period (Figure 4B).

During reperfusion of hearts following ischemia, insulin produced a 47% increase in the recovery of cardiac power in hearts perfused in the absence of fatty acids (Figure 3A and supplemental Table I; \( P < 0.05 \)). Insulin effects on glucose metabolism persisted during reperfusion, where a 97% increase in glycolysis was observed (Figure 4A; \( P < 0.05 \)) and a 160% increase in glucose oxidation (Figure 4B; \( P < 0.05 \)). Glucose oxidation during reperfusion recovered to the corresponding preischemic value in both the presence and absence of insulin. During reperfusion, a slight reduction in glycolysis in the insulin-treated hearts was observed, although this did not reach statistical significance (\( P = 0.08 \)). Although proton production was increased by 130% by insulin during aerobic perfusion (Figure 4C; \( P < 0.05 \)), insulin had no significant effect on proton production during reperfusion (Figure 4C).

**Ischemia and Reperfusion in the Presence of a High Concentration of Fatty Acids**

During the initial aerobic period, the presence of insulin did not significantly alter any parameters of mechanical function (supplemental Table II; \( P < 0.05 \)) but produced a 105% increase in glycolysis (Figure 5A; \( P < 0.05 \)) and a 274% increase in glucose oxidation (Figure 5B). However, during reperfusion in the presence of high fat, the cardioprotective effect of insulin seen in the absence of fatty acids was lost, and insulin now impaired recovery of cardiac power by 45%. (Figure 3B and supplemental Table II; \( P < 0.05 \)). During reperfusion, insulin increased glycolysis by 91% (Figure 5A; \( P < 0.05 \)) and glucose oxidation by 67% (Figure 5B; \( P < 0.05 \)). Unlike the no-fat group, glycolysis remained elevated during reperfusion, and glucose oxidation was suppressed by 44% compared with aerobic values (Figure 5B; \( P < 0.05 \)). As a consequence, proton production from glucose metabolism was increased by 92% in the insulin-treated hearts during reperfusion, compared with the no-insulin hearts (Figure 5C; \( P < 0.05 \)). The addition of insulin suppressed rates of fatty acid oxidation during both the aerobic period and the reperfusion period (Figure 5D; \( P < 0.05 \)).
Ischemia and Reperfusion Effects on AMPK Activity

In hearts perfused in the absence of fatty acids and frozen immediately following ischemia, there was a dramatic increase in AMPK activity (Figure 6A; \( P < 0.05 \)) that was inhibited by insulin (Figure 6A; \( P < 0.05 \)).

In the hearts perfused with high fat, insulin did not have any effect on AMPK activity either during the aerobic perfusion or postischemic reperfusion (Figure 6B). In addition, insulin was no longer able to blunt the dramatic rise in AMPK activity that occurred during ischemia in the presence of high fat (Figure 6B).

Discussion

A number of previous studies have examined the importance of substrate preference and its relationship to the recovery of cardiac function during reperfusion of severely ischemic hearts.\(^\text{16–18,24,30,31}\) Although insulin, fatty acids, and AMPK all have important effects on both function and metabolism following ischemia, the interaction among these 3 regulators of metabolism has not been examined. In this study, we used the isolated ejecting mouse heart, which is very sensitive to insulin, to examine the effect of insulin and fatty acids on myocardial metabolism, AMPK activity, and functional recovery during reperfusion following ischemia. Using this approach, we made a number of important findings. First, the ability of insulin to inhibit AMPK activity during both aerobic perfusion and during ischemia is dependent on the presence of fatty acids in the perfusate. Second, the cardio-
A novel finding of our study is that even though palmitate attenuates the ability of insulin to inhibit AMPK, other downstream signaling pathways of insulin are intact, because insulin activation of glycolysis, glucose oxidation, and phosphorylation of Ser473 of Akt (Figures 1 and 2 and supplemental Figure IIA) was not affected by the presence of fatty acid. This suggests that, in mouse heart, there is a fatty acid–dependent insulin-signaling pathway that modifies AMPK activity and a fatty acid independent insulin-signaling pathway that modifies myocardial metabolism.

These observations are of particular importance, as it has been shown that insulin can inhibit the ischemia-induced activation of AMPK, which would benefit the ischemic and reperfused myocardium by decreasing fatty acid oxidation. However, these previous studies were performed in the absence of fatty acids. It has also been shown that during reperfusion of ischemic hearts oxidative metabolism quickly recovers, but high rates of fatty acid oxidation result in low glucose oxidation rates attributable to the Randle cycle. This reduction in glucose oxidation is detrimental during reperfusion as it contributes to the severity of acidosis in the ischemic and reperfused heart. Ischemia-induced activation of AMPK contributes to the low glucose oxidation rates during reperfusion of ischemic hearts, secondary to stimulation of fatty acid oxidation. Insulin may attenuate these effects by inhibiting AMPK. However, we observed that insulin only inhibited AMPK activity in hearts perfused in the absence of fatty acids. Although insulin only induces a 15% inhibition in AMPK activity at the end of ischemia, we may be missing a larger effect as Beauloye et al showed that ischemia-induced AMPK activation peaks after 10 minutes of global ischemia and decreases with extended ischemic times. Therefore, insulin may suppress AMPK activation to a greater extent at 10 minutes of ischemia where peak activation occurs.

The cardioprotective effect of insulin has been well established in experimental studies. It has been suggested that this effect is attributable to either a positive inotropic effect or to a metabolic effect of insulin, such as the stimulation of anaerobic glycolysis to maintain basal ATP production and to switch the preferred oxidative substrate to glucose, which is a more oxygen-efficient substrate and does not produce the toxic intermediates of fatty acid oxidation. As seen in Figure 3, we have confirmed that insulin produces a cardioprotective effect in the absence of fatty acids, improving recovery of cardiac power by 47%. The beneficial effect of insulin during ischemia and reperfusion in the absence of fatty acids may be attributable to an acceleration of glycolysis, which would increase the amount of ATP being produced by the heart. In addition, proton production from glucose metabolism was not significantly elevated by insulin in hearts perfused in the absence of fatty acids. Therefore, the beneficial effects of insulin would be 2-fold, by increasing ATP production by the stimulation of glycolysis and glucose oxidation.

A surprising finding from this study is that in the presence of high fat, insulin impaired the recovery of mechanical function during reperfusion. Like the no-fat condition, insulin stimulated both glycolysis and glucose oxidation during the aerobic period and the reperfusion period. However, because insulin produced a greater stimulation of glycolysis than glucose oxidation, there was an increased in proton production from glucose metabolism during reperfusion (Figure 5). This may explain the detrimental effects of insulin during high-fat perfusion. As rates of fatty acid oxidation increase, this increases intracellular concentrations of acetyl–coenzyme A (acetyl-CoA), which is an allosteric inhibitor of pyruvate dehydrogenase (PDH). Indeed, the failure of insulin to inhibit AMPK activity may also contribute to this phenomenon. AMPK activation may lead to an acceleration of glucose uptake, thus supplying substrate for glycolysis, but also leads to phosphorylation of acetyl-CoA carboxylase, leading to a decrease in malonyl-CoA levels and an acceleration of fatty acid oxidation, which suppresses glucose oxidation by the abovementioned mechanism (supplemental Table II).

However, proton production during reperfusion in the high-fat plus insulin group was not increased significantly from values in the no-fat plus insulin group, despite dramatic differences in the effect on insulin on postischemic contractile function. The benefits of insulin on recovery of contractile function in the no-fat hearts may be related to an increase in energy supply to these hearts. The no-fat group may be energy starved and energetically compromised; thus, energy supply would be a better determinant of postischemic contractile function than proton production. Insulin decreased the AMP/ATP ratio during aerobic perfusion and increased ATP at the end of reperfusion in the no-fat group (supplemental Table III). However, in the presence of high fat, insulin did not change the AMP/ATP ratio during either aerobic perfusion or during reperfusion.

During the preparation of this report, a study by Horman et al was published suggesting that insulin may inhibit AMPK
via Akt phosphorylation of Ser485/491 on the α subunit of AMPK, corresponding with a previous report that Akt activity negatively correlates with AMPK Thr172 phosphorylation.36,37 Contradictory results were obtained in the present study. The addition of insulin produced an increase in phosphorylation of Akt at Ser473, which is indicative of Akt activity. Despite this apparent activation of this insulin-signaling pathway, there was no downstream inhibition of AMPK in the no fat palmitate group. This difference may be attributable to the use of a supraphysiological insulin concentration of 100 nmol·L⁻¹ in the Horman study, compared with a more physiological concentration (0.6 nmol·L⁻¹) in the present study. The only clinical study to report arterial insulin concentrations after GIK administration was a study of coronary surgery patients with type 2 diabetes. High-dose GIK therapy increased insulin concentrations to 10.3 nmol·L⁻¹ following administration.38

Considerable interest has also focused on GIK in both AMI and cardiac surgery patients.3,4 However, the recent large CREATE trial did not show GIK effectiveness. It is possible that the discrepant results may be related to differences in plasma fatty acid levels in the patient population. Despite the fact that some of the benefits of GIK have been attributed to a lowering of plasma free fatty acids, previous trials have not determined what effect GIK has on plasma fatty acids levels post–myocardial infarction or post surgery. In addition, the time of administration of GIK may play a key role in its efficacy, as the only group in the CREATE-ECLA trial with evidence of a trend to improvement was the population that received GIK before percutaneous coronary intervention, and this did reach statistical significance in a more recent trial.4,39

In summary, this study demonstrates that the cardioprotective effect of insulin and its ability to modify AMPK activity in the isolated ejecting mouse heart is highly dependent on the presence of fatty acids present in the perfusate. However, other insulin-signaling pathways, such as modification of glucose metabolism, are still intact in the presence of clinically relevant high concentrations of fatty acids. These findings have important implications in the use of insulin therapy for the treatment of ischemic heart disease.

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Disclosures
None.

References
24. Lopaschuk GD, Wandbolt RB, Barr RL. An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic reperfusion of ischemic hearts. J Pharmacol Exp Ther. 1993;264:135–144.


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Data Supplement

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Short title: Fatty acids modify insulin actions in the heart.

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**Mechanical function measurements in isolated working mouse hearts**

Heart rate and aortic pressure (mmHg) were measured with a Gould P21 pressure transducer (Harvard Apparatus) connected to the aortic outflow line. Cardiac output and aortic flow (mL•min⁻¹) were measured with Transonic T206 ultrasonic flow probes in the preload and afterload lines, respectively. Coronary flow (mL•min⁻¹) was calculated as the difference between cardiac output and aortic flow. Cardiac power was calculated as the product cardiac output times LV developed pressure (aortic systolic pressure minus preload pressure).

**Measurement of glycolysis, and glucose oxidation**

Glycolysis and glucose oxidation were measured by perfusing hearts with [5-\( ^3 \)H/U-\( ^14 \)C] glucose respectively. The total myocardial \( ^3 \)H₂O production and \( ^14 \)CO₂ production were determined at 10 min intervals during the 10 to 30 min aerobic perfusion period and the 40 min period of reperfusion. To measure the rates of glycolysis from tritiated substrate, \( ^3 \)H₂O in perfusate samples was separated from \([ ^3 \)H]glucose and \([ ^14 \)C]glucose using a vapor transfer method. This method consists of adding 500 µL of water into a 5 mL scintillation vial, then placing a lidless 1.5 mL microcentrifuge tube inside the scintillation vial. A 200 µL buffer sample is then added to the microcentrifuge tube, and the scintillation vial is capped. Scintillation vials are then stored initially at 50 °C for 24 hours and then at 4 °C for 24 hours. Following storage, the microcentrifuge tube is removed, scintillation fluid (Ecolite, ICN) is added and the vials are counted in a liquid scintillation counter. Glucose oxidation rates were determined by quantitative measurement of \( ^14 \)CO₂ production including \( ^14 \)CO₂ released as a gas in the oxygenation chamber and \( ^14 \)CO₂ dissolved as \( \text{H}^{14} \text{CO}_3^- \) in perfusate. The gaseous \( ^14 \)CO₂, which exits
the perfusion system via an exhaust line, was trapped in hyamine hydroxide solution. The dissolved $^{14}$CO$_2$ as $^{14}$CO$_3^-$ was released and trapped on filter paper saturated with hyamine hydroxide in the central well of 25 mL stoppered flasks after perfusate samples were acidified by the addition of 1 mL of 9N H$_2$SO$_4$.

**Calculation of H$^+$ production from glucose utilization**

If glucose passes through glycolysis to lactate and the ATP so formed is hydrolyzed, a net production of 2 H$^+$ per molecule of glucose occurs.$^4,^5$ In contrast, if glycolysis is coupled to glucose oxidation, the net production of H$^+$ is zero. Therefore, the overall rate of H$^+$ production derived from glucose utilization was determined by subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by 2. This calculation of proton production has been previously validated, as in a previous study in isolated working rat hearts utilizing a similar metabolic approach, the pH$_i$ determination using $^{31}$P NMR correlated very well with our calculated values.$^6$

**AMPK Assay**

Activity was assayed by following the incorporation of $[^{32}$P]phosphate into a pseudo-substrate AMARA peptide (AMARAASAAALARRR). The assay was performed in 25 µL of assay buffer containing 40 mmol•L$^{-1}$ HEPES-NaOH (pH = 7), 80 mmol•L$^{-1}$ NaCl, 1 mmol•L$^{-1}$ EDTA, 0.02% (w/v) Brij-35, 1 mmol•L$^{-1}$ DTT, 200 µM ATP, 5 mmol•L$^{-1}$ MgCl$_2$, phosphatase inhibitors (Sigma) and protease inhibitors (Sigma). The assay was performed at 30 °C for 5 min and was terminated by the addition of H$_3$PO$_4$ (1% v/v, final concentration). 15 µL aliquots were removed and spotted on a
Unifilter P81 (Whatman) 96-well filterplate. Each well was then washed 10 times with 300 µl of 1% (v/v) H₃PO₄, and 2 times with methanol. The filterplate was dried, and 50 µl of MicroScint PS scintillant (PerkinElmer Life Sciences) was added to each well and was subsequently counted in a MicroBeta Trilux (Wallac) scintillation counter.

**Western blotting**

Equal amounts of cleared homogenates were separated by SDS-PAGE and transferred to nitrocellulose membranes. These membranes were probed with anti-AMPK antibody (Cell Signaling Technologies), anti-phosphoSer-485/491 AMPK antibody (Cell Signaling Technologies), anti-Akt antibody (Cell Signaling Technologies) or anti-Ser-473 Akt antibody (Cell Signaling Technologies), followed by a horseradish peroxidase-couple goat anti-rabbit secondary antibody (Jackson ImmunoResearch).

**High Energy Phosphate Measurements**

The tissue content of AMP, ADP, ATP, Cr and PCr were measured in neutralized perchloric acid extracts of frozen tissue by HPLC, as previously described.⁷,⁸
**Group #1: Aerobic Protocol**

- **Group #1a**: 0 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin
- **Group #1b**: 0.2 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin
- **Group #1c**: 1.2 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin

**Group 2: Ischemia-Reperfusion Protocol**

- **Group #2a**: 0 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin
- **Group #2b**: 1.2 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin
- **Group #2c**: 0 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin
- **Group #2d**: 1.2 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin
- **Group #2e**: 0 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin
- **Group #2f**: 1.2 mmol•L⁻¹ Palmitate, +/- 100 µU•mL⁻¹ Insulin

**Supplemental figure 1**: Isolated working mouse heart perfusion groups.
Supplemental figure 2: Insulin produces a robust increase in Ser-473 phosphorylation of Akt, however has no effect on phosphorylation of AMPK on Ser-485/491.
A) Western blot of P-Ser-473 of Akt and total Akt (n = 3 per group) and B) Western blot of P-Ser-485/491 of AMPK and total AMPK (n = 6 per group). * represents $P < 0.05$, significantly different from corresponding no insulin group.
**Supplemental Table 1.** The effect of insulin on parameters of mechanical function during aerobic reperfusion of ischemic hearts perfused in the absence of fatty acids.

<table>
<thead>
<tr>
<th>Insulin</th>
<th>Aerobic Perfusion</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 13)</td>
<td>(n = 11)</td>
</tr>
<tr>
<td>Heart Rate (beats•min⁻¹)</td>
<td>294 ± 8</td>
<td>296 ± 13</td>
</tr>
<tr>
<td>Peak Systolic Pressure (mmHg)</td>
<td>72 ± 1</td>
<td>73 ± 2</td>
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<tr>
<td>Coronary Flow (mL•min⁻¹)</td>
<td>3.2 ± 0.3</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Cardiac Output (mL•min⁻¹)</td>
<td>11.1 ± 0.4</td>
<td>12.7 ± 0.5</td>
</tr>
<tr>
<td>Cardiac Power (mJoule•min⁻¹)</td>
<td>89 ± 4</td>
<td>103 ± 6</td>
</tr>
</tbody>
</table>

Hearts were perfused with 5 mmol•L⁻¹ glucose in the absence of 1.2 mmol•L⁻¹ palmitate, and in the presence or absence of 100 µU/ml insulin. Hearts were subjected to 16 min global no-flow ischemia and 40 min reperfusion.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

Differences were determined using a 2-way ANOVA with a Bonferroni posthoc test.
**Supplemental Table 2.** The effect of insulin on parameters of mechanical function during aerobic reperfusion of ischemic hearts perfused in the presence of high fat.

<table>
<thead>
<tr>
<th>Insulin</th>
<th>Aerobic Perfusion</th>
<th>Reperfusion</th>
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<tbody>
<tr>
<td></td>
<td>- (+) (n = 24)</td>
<td>+ (+) (n = 22)</td>
</tr>
<tr>
<td>Heart Rate (beats•min⁻¹)</td>
<td>327 ± 7</td>
<td>322 ± 11</td>
</tr>
<tr>
<td>Peak Systolic Pressure (mmHg)</td>
<td>66 ± 1</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>Coronary Flow (mL•min⁻¹)</td>
<td>3.6 ± 0.3</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>Cardiac Output (mL•min⁻¹)</td>
<td>11.3 ± 0.6</td>
<td>9.9 ± 0.5</td>
</tr>
<tr>
<td>Cardiac Power (mJoule•min⁻¹)</td>
<td>83 ± 5</td>
<td>74 ± 4</td>
</tr>
</tbody>
</table>

Hearts were perfused with 5 mmol•L⁻¹ glucose in the presence of 1.2 mmol•L⁻¹ palmitate, and in the presence or absence of 100 µU/ml insulin. Hearts were subjected to 18 min global no-flow ischemia and 40 min reperfusion.

* represents \( P < 0.05 \), significantly different from corresponding aerobic perfusion group. Differences were determined using a 2-way ANOVA with a Bonferroni posthoc test.
**Supplementary Table 3.** The effect of insulin on adenine nucleotides during aerobic perfusion, and aerobic reperfusion in isolated working mouse hearts in the absence of fatty acids.

<table>
<thead>
<tr>
<th>Insulin</th>
<th>Aerobic Perfusion</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>AMP (µmol•g dry wt⁻¹)</th>
<th>ADP (µmol•g dry wt⁻¹)</th>
<th>ATP (µmol•g dry wt⁻¹)</th>
<th>AMP/ATP</th>
<th>ADP/ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1.7± 0.2</td>
<td>6.8 ± 0.7</td>
<td>21.1 ± 1.1</td>
<td>0.084 ± .011</td>
<td>0.373 ± .010</td>
</tr>
<tr>
<td>+</td>
<td>1.0 ± 0.1*</td>
<td>6.9 ± 0.7</td>
<td>23.4 ± 0.8</td>
<td>0.043 ± .002*</td>
<td>0.359 ± .005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4 ± 0.1</td>
<td>16.3 ± 1.3#</td>
<td>0.093 ± .006</td>
<td>0.423 ± .029</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6 ± 0.1#</td>
<td>21.4 ± 0.7*</td>
<td>0.074 ± .001#</td>
<td>0.320 ± .016*</td>
</tr>
</tbody>
</table>

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding High Fat group.

# represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.
**Supplementary Table 4.** The effect of insulin on adenine nucleotides during aerobic perfusion and aerobic reperfusion in isolated working mouse hearts in the presence of High Fat.

<table>
<thead>
<tr>
<th>Insulin</th>
<th>Aerobic Perfusion</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>AMP</td>
<td>2.0 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>(µmol•g dry wt⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>8.7 ± 0.4</td>
<td>6.6 ± 0.1*</td>
</tr>
<tr>
<td>(µmol•g dry wt⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>23.6 ± 1.3</td>
<td>18.3 ± 0.4*</td>
</tr>
<tr>
<td>(µmol•g dry wt⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP/ATP</td>
<td>0.087 ± .003</td>
<td>0.092 ± .003</td>
</tr>
<tr>
<td>ADP/ATP</td>
<td>0.374 ± .007</td>
<td>0.363 ± .008</td>
</tr>
</tbody>
</table>

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding High Fat group.

*# represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.
Supplemental Reference List


(2) Lopaschuk GD, Wambolt RB, Barr RL. An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic reperfusion of ischemic hearts. *J Pharmacol Exp Ther.* 1993; 264:135-44.


