Insulin Inhibition of 5′Adenosine Monophosphate–Activated Protein Kinase in the Heart Results in Activation of Acetyl Coenzyme A Carboxylase and Inhibition of Fatty Acid Oxidation

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Acetyl coenzyme A (CoA) carboxylase (ACC) is an important regulator of fatty acid oxidation in the heart, since it produces malonyl CoA, a potent inhibitor of mitochondrial fatty acid uptake. Under conditions of metabolic stress, 5′adenosine monophosphate–activated protein kinase (AMPK), which is highly expressed in cardiac muscle, can phosphorylate and decrease ACC activity. In this study, we determined if fatty acid oxidation in the heart could be regulated by insulin, due to alterations in AMPK regulation of ACC activity. Isolated working rat hearts were perfused with Krebs-Henseleit solution containing 11 mmol/L glucose, 0.4 mmol/L [9,10-^{3}H]palmitate, and either 100 μU/mL insulin or 1,000 μU/mL insulin. Increasing insulin concentration resulted in a decrease in fatty acid oxidation rates ($P < .05$), a decrease in AMPK activity ($P < .05$), and an increase in ACC activity ($P < .05$) compared with the low-insulin group. A negative correlation was observed between AMPK and ACC activity ($r = -.76$). We conclude that insulin, acting through inhibition of AMPK and stimulation of ACC, is capable of inhibiting myocardial fatty acid oxidation.

THE ENERGY NECESSARY to maintain contractile work in the heart is primarily derived from oxidation of carbohydrates and fatty acids. Under aerobic conditions, fatty acid oxidation accounts for the majority of adenosine triphosphate (ATP) produced by the heart (~60%), with the remainder primarily originating from glucose oxidation.[1,2] Insulin can increase glucose usage by the heart both by increasing myocardial glucose uptake and by decreasing the extracellular fatty acid concentration. Whether insulin can directly alter fatty acid oxidation in the heart is less clear.

Alterations in cytoplasmic malonyl coenzyme A (CoA) levels are an important regulator of myocardial fatty acid oxidation by virtue of an ability to inhibit carnitine palmitoyltransferase 1 (CPT 1).[3,4] Inhibition of CPT 1 prevents fatty acids from gaining access to the mitochondrial matrix, thus decreasing β-oxidation. Malonyl CoA is the product of acetyl CoA carboxylase (ACC), the activity of which plays an important role in regulating carbohydrate and fatty acid metabolism.[5-8] In the heart, a 280-kd isoenzyme is predominantly expressed, with a 265-kd isoenzyme expressed to a lesser extent.[5,9,10]

In lipogenic tissues (such as liver and adipose tissue), regulation of ACC is manifested through a cycle of phosphorylation-dephosphorylation (see Kim et al[11] for a review). The degree of phosphorylation is thought to be reflected by the dependence of enzyme activity on the allosteric activator citrate. The hormonal control of ACC in liver and adipose tissue has been studied extensively, but less is known regarding hormonal control of the 280-kd isoform of ACC. We recently showed that under conditions of metabolic stress (ie, myocardial ischemia) cardiac 5′adenosine monophosphate–activated protein kinase (AMPK) can phosphorylate and inhibit ACC activity.[8,12] Whether AMPK regulates fatty acid oxidation in the absence of stress is not known. In the liver, insulin activates the 265-kd isoform of ACC[13] through a dephosphorylation mechanism involving inhibition of AMPK.[14] Awan and Saggerson[6] have recently suggested that insulin can acutely increase the levels of malonyl CoA and reduce fatty acid oxidation in cardiac myocytes, although the potential involvement of AMPK in this process was not investigated. We therefore directly examined the effects of insulin on AMPK activity, ACC activity, and palmitate oxidation rates in isolated working rat hearts.

MATERIALS AND METHODS

Materials

[9,10-^{3}H]palmitic acid was obtained from NEN (Wilmington, DE). Bovine serum albumin (fraction V) was obtained from Boehringer Mannheim (Indianapolis, IN). Aqueous Counting Scintillant and Eco-Lite Scintillant were obtained from Amersham (Oakville, Ontario, Canada). Insulin was obtained from Connaught Laboratories (Toronto, Ontario, Canada). Protein phosphatase 2A was purchased from Upstate Biotechnology (Lake Placid, NY). ECL Western blotting detection reagents were purchased from Amersham International (Amersham, UK). Peroxidase-labeled streptavidin was purchased through Mandel Scientific from Kirkegaard & Perry Laboratories (Gaithersburg, MD). For Western blotting, Trans-Blot Transfer Medium (pore nitrocellulose membrane, 0.45 μm) was obtained from BioRad (Richmond, CA). X-ray films (X-OMAT AR) were purchased from Eastman Kodak (Rochester, NY). All other chemicals were obtained from Sigma Chemical (St Louis, MO).

Heart Perfusions

Hearts were obtained from adult male Sprague-Dawley rats (250 to 300 g) that were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally). Isolated working hearts were cannulated as previously described[8] and perfused at 11.5 mm Hg left atrial preload and 80 mm Hg aortic afterload. Spontaneously beating hearts were used throughout the studies. All hearts were perfused with Krebs-Henseleit solution containing 0.4 mmol/L [9,10-^{3}H]palmitate prebound to 3% bovine serum albumin, 11 mmol/L glucose, and 2.5 mmol/L free Ca^{2+} for a period of 40 minutes. Hearts referred to as "control" were perfused in the presence of 100 μU/mL insulin, and hearts referred to as "insulin" were perfused in the presence of 1,000 μU/mL. A concentra-

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tion of 100 μU/mL insulin was used in the control hearts, since under these conditions glucose uptake is not limiting in any of the experimental groups.[1] This ensured that any effects of insulin on fatty acid oxidation did not occur secondary to an increase in glucose uptake and metabolism.

Palmitate Oxidation Measurements

Cumulative and steady-state palmitate oxidation rates were determined from ^3H_2O production of hearts perfused with [^{3}H]palmitate, as described previously.[8] Buffer samples were taken at 10-minute intervals over the course of the perfusion, as were recordings of mechanical function.

Biochemical Analysis

At the end of the perfusions, heart ventricles were quickly frozen with tongs cooled to the temperature of liquid N_2. ACC and AMPK were extracted using 6% polyethylene glycol (PEG) 8000 from frozen heart tissue as previously described.[8] The CO_2 fixation technique was used to measure ACC activity in the PEG fraction, as described previously.[8] To determine the phosphorylation status of ACC, the enzyme activity was measured in the presence or absence of NaF and NaPPi.[8] Western blot analysis of ACC was performed as previously described.[8] AMPK activity was measured according to the method of Davies et al.,[13] with slight modification.[8]

Determination of CoA Esters

CoA esters were extracted as described previously.[5] Following extraction with 6% PCA, CoA ester levels were measured by high-performance liquid chromatography (HPLC) analysis.[5] Separation was performed on a Beckman System Gold with UV detector 167. Each sample (100 μL each) was run through a precolumn cartridge (C18; size 3 cm, 7 μm) and a Microsorb short-one column (type C18; particle size 3 μm and size 4.6 × 100 mm). Absorbance was set at 254 nm and flow rate at 1 mL/min. A gradient was initiated using two buffers: buffer A consisted of 0.2 mmol/L NaH_2PO_4 (pH 5.0), and buffer B was a mixture of 0.25 mol/L NaH_2PO_4 and acetonitrile (pH 5.0) in a ratio of 80:20 (vol/vol). Buffers were filtered using a filter-pure Nylon-66 filter membrane (Pierce Chemical, Rockford, IL). Initial conditions (97% A and 3% B) were maintained for 2.5 minutes and were changed thereafter to 18% B over 5 minutes using Beckman's curve 3. At 15 minutes the gradient was changed linearly to 37% B over 3 minutes and subsequently to 90% B over 17 minutes. At 42 minutes, the composition was returned linearly to 3% B over 0.5 minutes, and at 50 minutes, column equilibration was complete. Peaks were integrated by the Beckman System Gold software package.

Statistical Analysis

Data are presented as the mean ± SEM. The unpaired Student *t* test was used as appropriate to determine statistical significance between sample populations. *P* less than .05 was regarded as significant.

RESULTS

Insulin Effects on Heart Function

Heart function was monitored continuously over the course of the perfusions in isolated working rat hearts. Insulin added at a concentration of 1,000 μU/mL had no effect on any of the indices of heart function compared with hearts perfused with 100 μU/mL insulin (control). Heart rate was 298 ± 11 versus 288 ± 6 beats/min in the control group and insulin group, respectively, and peak systolic blood pressure was 120 ± 3 and 130 ± 4 mm Hg, respectively. Cardiac output was also similar in the control and insulin hearts (65 ± 4 *v* 71 ± 3 mL/min, respectively). Therefore, any insulin changes in energy metabolism in our studies cannot be attributed to differences in metabolic demand by the heart.

Insulin Effects on Palmitate Oxidation

Rates of palmitate oxidation are shown in Table 1. Cumulative palmitate oxidation rates were linear between 10 and 40 minutes of the perfusions under all study conditions (data not shown). Increasing insulin levels resulted in a significant decrease in palmitate oxidation rates relative to the control hearts. It should be noted that in this study the control hearts already had 100 μU/mL insulin present, and insulin was increased to 1,000 μU/mL. This ensured that glucose uptake would not be limiting in any of the experimental conditions, and that the effects of insulin on fatty acid oxidation did not occur secondary to an increase in glucose uptake or metabolism. Indeed, under identical conditions, we have shown that glucose oxidation is not stimulated by increasing insulin concentration (data not shown).

Insulin Effects on ACC Activity

Table 2 shows ACC activity measured in PEG extracts obtained from control and insulin hearts. If ACC was isolated and measured in the presence of NaF and NaPPi, which preserves the phosphorylated state seen in vivo, increasing insulin levels resulted in a 1.9-fold activation of myocardial ACC activity (Table 2).

To obtain information with regard to the phosphorylation state of ACC, PEG extracts were also prepared in the absence of NaF and NaPPi and ACC activity was measured (Table 2). Under these conditions, an increase in ACC activity was seen in all hearts. This is thought to be due to a dephosphorylation and activation of ACC during isolation. The smallest increase in ACC activity was observed in hearts perfused with insulin (9.3%).

To determine whether any of the differences in enzyme activity could be attributed to changes in the protein expression in response to exposure of the hearts to high insulin, Western blotting was performed on the PEG extracts. There was no apparent change in the relative amounts of either the 280- or 265-kd isoenzymes in response to perfusion under control or insulin conditions (data not shown).

To determine whether changes in ACC activity would be reflected in changes in overall tissue levels of malonyl CoA, CoA esters were isolated from frozen ventricular tissue, separated, and quantified using HPLC. Perfusing hearts in the

Table 1. Insulin Effects on Steady-State Rates of Fatty Acid Oxidation in Isolated Working Hearts

Perfusion Condition	Palmitate Oxidation Rate (nmol · g dry weight^{-1} · min^{-1})
Control	723 ± 67
Insulin	534 ± 28*

NOTE. Values are the mean ± SEM of 6 hearts in each group. Palmitate oxidation rates were determined by quantitative collection of ^3H_2O from [9,10-^{3}H]-palmitate.

*Significantly different from control.

Table 2. Insulin Effects on ACC Activity in Isolated Working Hearts

Perfusion Condition	ACC Activity (nmol · min^{-1} · mg protein^{-1}) Isolated With NaF, NaPPi	Isolated Without NaF, NaPPi	% Change
Control	4.92 ± 0.8	6.29 ± 1.2	27.8
Insulin	9.49 ± 1.6*	10.38 ± 0.7*	9.3

NOTE. Values are the mean ± SEM of 6 hearts in each group. ACC activity was determined in 6% PEG precipitates (isolated in the presence or absence of NaF and NaPPi) from frozen, pulverized ventricular tissue.

*Significantly different from control.

presence of 1,000 μU/mL insulin resulted in no significant differences in tissue levels of malonyl CoA (42.1 ± 8 *v* 37.9 ± 7 nmol · g dry weight^{-1} in control and insulin groups, respectively). However, it should be pointed out that compartmentalization of malonyl CoA probably exists, and the value of total tissue levels of malonyl CoA as a predictor of malonyl CoA available to inhibit CPT 1 remains uncertain.

Insulin Effects on AMPK Activity

To determine whether the alterations in ACC activity could be explained by changes in AMPK activity, we also measured AMPK activity in the control and insulin hearts (Fig 1). As expected, addition of 200 μmol/L AMP in vitro resulted in a significant increase in AMPK activity in all hearts. In the insulin group, a significant decrease in AMPK activity was observed compared with control hearts. When saturating amounts of AMP were added to the incubation medium, AMPK remained inhibited in the insulin group. This is consistent with a decreased phosphorylation and inactivation of AMPK in the insulin group.

If tissue extracts were pretreated with protein phosphatase 2A, AMPK activity was completely abolished in all hearts (data not shown). This also suggests that cardiac AMPK activity is indeed under phosphorylation control.

The correlation between ACC and AMPK activity in control and insulin hearts is shown in Fig 2. In these groups, a significant negative correlation was found between ACC and AMPK activity ($r = -.76$). Hearts treated with a high dose of insulin showed higher ACC activity that was associated with lower AMPK activity, while control hearts showed a lower ACC activity and higher AMPK activity.

DISCUSSION

Within the cardiac muscle cell, the integrated regulation of contractile activity and the pathways associated with energy substrate metabolism remains, to a large extent, undefined. The influence of various hormones on these parameters is even less clearly understood. In this study, we examined the influence of insulin on energy substrate utilization and on the enzymes thought to be important in regulating these metabolic pathways, namely ACC and AMPK. The conclusions that can be made from this study are that insulin can inhibit AMPK activity in the intact heart, and insulin inhibition of AMPK is associated with an increase in ACC activity and a decrease in fatty acid oxidation rates.

Treatment of the hearts with insulin (1,000 μU/mL) resulted in a significant reduction in steady-state rates of palmitate oxidation (Table 1). This finding is entirely consistent with a prior study that suggested that insulin may decrease myocardial palmitate oxidation while having no effect on free fatty acid extraction.[15] We propose that in the heart, an increase in myocardial insulin levels results in dephosphorylation and

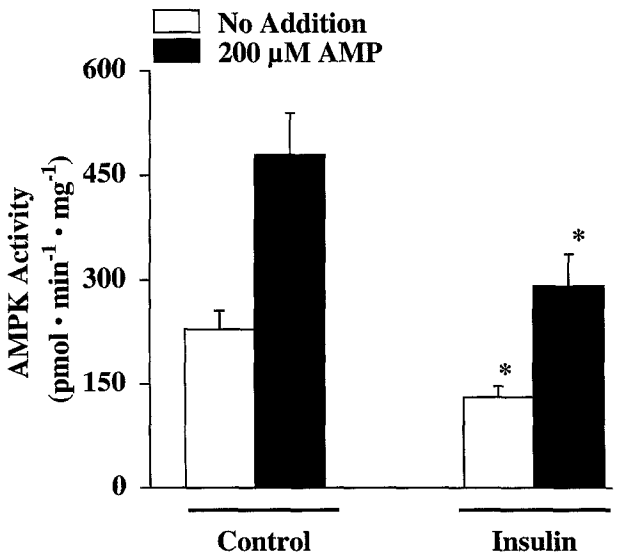

Fig 1. Insulin effects on AMPK activity in control and insulin hearts. Values represent the mean ± SEM of 6 hearts in each group. Assays were conducted in the absence of added AMP or in the presence of 200 μmol/L AMP. *Significantly different from control.

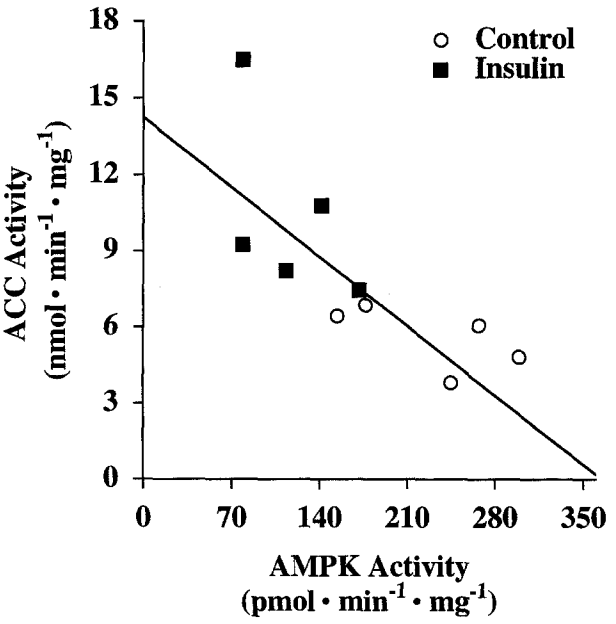

Fig 2. Correlation between myocardial ACC and AMPK activity. Values are plotted for ACC and AMPK activity in control and insulin hearts. Hearts were frozen at the end of the perfusion protocol, and enzyme activity was measured in 6% PEG extracts.

inactivation of AMPK. The reduction in AMPK activity decreases the phosphorylation of ACC, resulting in activation of the enzyme. This increased ACC activity gives rise to enhanced cytoplasmic levels of malonyl CoA, the product of the ACC reaction. Since malonyl CoA is a potent inhibitor of CPT 1, the enzyme considered rate-limiting for fatty acid uptake into the mitochondria, fatty acid oxidation is inhibited. As a result, myocardial fatty acid oxidation rates decrease as insulin levels increase.

In this study, we did not observe any major change in total malonyl CoA levels in the heart following insulin treatment. However, it should be recognized that it is unlikely that all the malonyl CoA present in the heart is accessible to CPT 1. This is because total measured levels of malonyl CoA in the heart range from 5 to 30 nmol/g dry weight,[5,7] which, if all cytosolic, would roughly translate into a cytoplasmic concentration of 2 to 15 μmol/L. However, the IC_{50} for cardiac CPT 1 is in the 30 to 50-nmol/L range. Therefore, malonyl CoA must be compartmentalized in the heart and cannot always be accessible to CPT 1, or the heart would not oxidize fatty acids (which clearly is not the case). Unfortunately, it is technically difficult (if not impossible) to obtain an accurate measure of the actual cytoplasmic levels of malonyl CoA accessible to CPT 1. While we have observed a correlation between total tissue levels of malonyl CoA and fatty acid oxidation rates in some of our studies,[5,7,8] this relationship does not persist under all experimental conditions. However, we have observed a close inverse relationship between the activity of cytoplasmic ACC (which produces malonyl CoA) and fatty acid oxidation rates.[5,7,8] Therefore, the relationship between ACC activity (which is a cytoplasmic enzyme) and fatty acid oxidation in the diabetic rat heart appears to provide a better correlation than the relationship between malonyl CoA and fatty acid oxidation.

We have previously proposed an integral role for ACC in regulating the balance between myocardial carbohydrate and fatty acid metabolism.[5] Using dichloroacetate, an agent that causes a net activation of the pyruvate dehydrogenase complex, we found a stimulation of myocardial glucose oxidation at the expense of fatty acid oxidation. This was accompanied by an increase in the levels of malonyl CoA, which were inversely correlated with the rates of fatty acid oxidation and positively correlated with acetyl CoA levels. Based on these previous results,[5] we propose that an increase in intramitochondrial acetyl CoA stimulates acetylcarnitine synthesis by the intramitochondrial carnitine acetyltransferase. This then results in an increase in carnitine acetylcarnitine translocase, which shuttles acetyl groups out of the mitochondria into the cytosol. Once re-esterified to CoA, the acetyl units act as a substrate for ACC, which produces malonyl CoA and inhibits fatty acid oxidation at the level of CPT 1. Since glucose oxidation was not stimulated by insulin under the experimental conditions used, we propose that insulin does not decrease fatty acid oxidation by increasing this shuttle pathway, but rather by directly activating ACC.

The mammalian ACC enzyme is a large, complex multifunctional enzyme that catalyzes the carboxylation of acetyl CoA to form malonyl CoA.[11] Extensive characterization has been made of the 265-kd isoenzyme at the level of both the protein and the gene. In tissues such as the liver, ACC can undergo rapid reversible phosphorylation resulting in inhibition of enzyme activity.[11] The ACC-265 protein contains a number of sites that can be phosphorylated by at least seven different kinases. One of the most important kinases involved in the phosphorylation-induced inhibition of ACC activity is AMPK, which phosphorylates hepatic ACC on Ser-79.[16] AMPK is activated by AMP through either a direct allosteric activation,[17] increased activity of an upstream kinase kinase,[18] facilitation of AMPK kinase phosphorylation of AMPK,[19] or inhibition of dephosphorylation of AMPK.[20] A direct phosphorylation of AMPK is supported by our results, which showed that regardless of the perfusion condition, AMPK activity was markedly reduced in vitro either following isolation under conditions that favor dephosphorylation of the enzyme or after incubation with protein phosphatase 2A. AMPK is a multisubstrate kinase that responds to alterations in the energy status of the cell and thus has been termed a "stress kinase." Indeed, in rat hepatocytes, induction of cellular stress by either substrate depletion[21,22] or heat shock[23] results in activation of AMPK, which serves to switch off ATP-consuming biosynthetic pathways such as fatty acid and cholesterol synthesis in an attempt to preserve ATP for more essential processes such as maintenance of ion homeostasis. Our results would suggest that in the heart, AMPK also acts to increase or decrease fatty acid oxidation in times of high or low metabolic demand, respectively. They also demonstrate that this can occur in the absence of metabolic stress. Inhibition of AMPK is an attractive mechanism by which insulin decreases fatty acid oxidation in parallel with its well-described effects on glucose metabolism.

Phosphorylation of hepatic ACC by AMPK is accompanied by marked decreases in the ACC-265 enzyme activity. Until recently, it was not known whether this same phenomenon occurred in the heart. The heart does express a significant amount of mRNA encoding AMPK,[24-26] which is primarily the α2 catalytic subunit of AMPK.[27] We have recently shown that myocardial ACC activity is inversely correlated with both AMPK activity and levels of fatty acid oxidation in hearts subjected to ischemia followed by reperfusion,[8,12] providing strong evidence for a role of AMPK in regulating myocardial fatty acid oxidation via phosphorylation and thus inhibition of ACC. Indeed, an inverse correlation was found between myocardial ACC and AMPK activity in control hearts and hearts treated with high-dose insulin.

The heart contains a 265-kd isoenzyme in addition to a 280-kd isoenzyme that is predominantly expressed.[5] Unfortunately, unlike the 265-kd isoenzyme of ACC, the 280-kd ACC has not been extensively characterized at either the protein or the gene level. While it is not known whether the phosphorylation sites on ACC-265 are also present in ACC-280, it has been suggested that the ACC-280 in liver is more readily phosphorylated by the catalytic subunit of cAMP-dependent protein kinase.[28] In this study, we did not attempt to correlate these changes in phosphorylation state to alterations in ACC-280 enzyme activity. However, our laboratory has also shown indirectly[12] and directly (Kudo N, et al, unpublished data, 1996)

that phosphorylation of ACC inhibits enzyme activity. A recent study by Wang et al[29] has also shown that the 280-kd isoform of ACC is under phosphorylation control.

In this study, insulin concentrations were increased from 100 to 1,000 μU/mL. At the higher concentration, insulin could potentially be acting through a type 1 IGF receptor rather than the insulin receptor. The studies as performed do not allow us to exclude this possibility.

In summary, our data suggest that insulin can decrease fatty acid oxidation in the heart, secondary to an inhibition of AMPK and an increase in ACC activity. Using an indirect measure of ACC phosphorylation, we show that the ability of insulin to stimulate ACC activity is associated with a dephosphorylation of the enzyme, supporting the role of AMPK in this process.

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