Phosphorylation of Skeletal Muscle Acetyl-CoA Carboxylase by AMPK Enhances Palmitoyl-CoA Inhibition

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PHOSPHORYLATION OF SKELETAL MUSCLE ACETYL-COA CARBOXYLASE
BY AMPK ENHANCES PALMITOYL-COA INHIBITION

by

Dustin S. Rubink

A thesis submitted to the faculty of

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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate’s graduate committee, I have read the thesis of Dustin S. Rubink in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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Acetyl-CoA carboxylase (ACC) catalyzes the formation of malonyl-CoA, which in turn controls the rate of fatty acid metabolism. ACC beta or 2 has been shown to be localized on the mitochondria in close proximity to carnitine palmitoyl transferase 1 (CPT-1), the enzyme responsible for the influx of acyl-CoA into the matrix where beta oxidation takes place. CPT-1 is inhibited by malonyl-CoA produced by ACC. It has been well documented that AMP activated kinase (AMPK) when activated phosphorylates and inactivates ACC. ACC is controlled allosterically by citrate, which activates, and by palmitoyl-CoA, which inhibits. In this study, we asked the question, “Does phosphorylation by AMPK effect the inhibition of ACC by palmitoyl-CoA?” ACC was isolated and then subjected to phosphorylation and activity was measured in varying concentrations of acetyl-CoA and citrate. Phosphorylation reduced the substrate (acetyl-CoA) saturation activity curves for ACC at all levels of palmitoyl-CoA. The Ki for
palmitoyl-CoA inhibition of ACC was reduced from $1.7 \pm 0.25 \, \mu M$ to $0.85 \pm 0.13 \, \mu M$ (p<0.05) as a consequence of phosphorylation. In addition the citrate activation curves for ACC were greatly reduced in the presence of palmitoyl-CoA. The data show that skeletal muscle ACC or ACC-beta is more potently inhibited by palmitoyl-CoA after phosphorylation by AMPK. During long-term exercise when AMPK is activated and muscle palmitoyl-CoA is elevated this may contribute to the low malonyl-CoA and increased fatty acid oxidation.

Key Words: acetyl-CoA, exercise, fatty acid oxidation, malonyl-CoA
ACKNOWLEDGEMENTS

These studies were supported by NIH Grant AM41438. The authors thank Dr. D. Grahame Hardie for the AMPK used in the phosphorylation studies. Thanks are also given to Dr. William W. Winder for mentoring Dustin S. Rubink during the completion of this project.
# TABLE OF CONTENTS

- TITLE PAGE ..............................................................................................................i
- GRADUATE COMMITTEE APPROVAL .................................................................ii
- FINAL READING APPROVAL AND ACCEPTANCE .............................................iii
- ABSTRACT ...............................................................................................................iv
- ACKNOWLEDGEMENTS .........................................................................................vi
- TABLE OF CONTENTS ..........................................................................................vii
- LIST OF FIGURES ...................................................................................................viii
- BODY OF THE WORK ............................................................................................1-28
  - INTRODUCTION ..................................................................................................1
  - MATERIALS AND METHODS ............................................................................3
  - RESULTS ............................................................................................................7
  - DISCUSSION .......................................................................................................9
  - REFERENCES .....................................................................................................15
LIST OF FIGURES

FIGURE 1: Western Blot Time Analysis ...............................................................23
FIGURE 2: ACC Activity Curves .................................................................24
FIGURE 3: Apparent Vmax ...........................................................................25
FIGURE 4: Citrate Activation Curves ..............................................................26
FIGURE 5: Activity at Two Citrate Concentrations .........................................27
FIGURE 6: Inhibition Curves .........................................................................28
INTRODUCTION

Acetyl-CoA carboxylase (ACC) controls an important regulatory step in fatty acid metabolism (25). ACC catalyzes carboxylation of acetyl-CoA to form malonyl-CoA (5,6,20). There are two major isoforms of ACC in mammals, including humans (2). ACC alpha, found predominately in the liver and adipose tissue, catalyzes a rate limiting step in fatty acid biosynthesis. The product malonyl-CoA is the substrate for fatty acid synthase (FAS) in lipogenic tissues. There has been increasing evidence that ACC beta found in muscle, considered a non-lipogenic tissue, is involved in controlling the rate of fatty acid oxidation(5-7,34,37,44-50). Here the malonyl-CoA formed inhibits carnitine palmitoyl transferase-1, the enzyme responsible for the regulation of the transport of fatty acids into the mitochondria by forming the carnitine derivative (22). In the mitochondrial matrix, the CoA derivative is regenerated by CPT-2 and can then be oxidized to acetyl-CoA by the enzymes of beta oxidation (22).

There is increasing evidence that ACC-beta has potential in therapeutically regulating metabolic diseases such as obesity and diabetes. This was demonstrated recently by creation of an ACC-beta -/- null mutant mouse phenotype (2). These mice had less fat mass and higher rates of fatty acid oxidation, while continuing to be fertile and have a normal life span(2). It has also been demonstrated that the null mutant mice had reduced obesity and delayed onset of insulin-insensitivity while being fed obesity inducing diets, specifically high fat or high carbohydrate diets (1).

There has been skepticism regarding the importance of malonyl-CoA in the regulation of fatty acid oxidation. This is due to the fact that there has been a lack of evidence of decreases in malonyl-CoA content in human muscle during exercise (19,27-
Recent data have made regulation of fatty acid oxidation by malonyl-CoA more feasible due to elucidation of the cellular localization of ACC beta (2). ACC beta is a larger protein (272 kD vs 265 kD) than ACC alpha due primarily to an N terminal extension that has a hydrophobic mitochondrial targeting sequence (1,9,18). ACC beta has been shown to be associated with the mitochondria by immunohistochemical techniques and confocal microscopy (2). The malonyl-CoA that is produced by the ACC beta is closely associated with the CPT-1 and can have an inhibitory effect without drastic changes to the total cellular content of malonyl-CoA (2). In rat the total muscle content of malonyl-CoA is higher than in human muscle, particularly in the fast-twitch red muscle fibers (8,13,27-28,32,47). Malonyl-CoA declines markedly in this tissue in response to exercise or electrically stimulated muscle contraction (17,31,33,46). Hindlimb perfusion studies demonstrate a good correlation between the rate of fatty acid oxidation and the muscle malonyl-CoA content (23,24,50).

ACC is allosterically regulated by citrate, which activates ACC, and also by palmitoyl-CoA, an inhibitor of ACC (6,25,40). There is also the phosphorylation / dephosphorylation by AMP activated protein kinase (AMPK) and acetyl CoA carboxylase phosphatase on specific serine residues (particularly ser 221) (10,14,51). AMPK has previously been shown to phosphorylate the muscle isoform of acetyl-CoA carboxylase (ACC beta) at the site equivalent to serine 79 in ACC-1 (ser221)(16,48,51). It has been demonstrated that AMPK is activated in response to muscle contraction (17,30,42,48). It has also been shown that ACC beta is phosphorylated and inactivated in exercising or electrically stimulated muscle (13,17,30,31,33,38,42). Phosphorylation of ACC beta by AMPK results in a shift of the citrate activation curve to the right, making the enzyme
insensitive to allosteric activation at physiological citrate concentrations (30,48,51). This coupled with removal of malonyl-CoA by malonyl-CoA decarboxylase results in a marked decrease in malonyl-CoA concentration in the muscle (35,37,38,48). This is thought to remove inhibition of CPT1, allowing fatty acid oxidation to proceed as exercise continues.

In addition to activation by citrate, ACC has been shown to be inhibited by palmitoyl-CoA (29,41). The content of long chain acyl-CoA in muscle increases during long-term exercise (43). No data is available regarding the effect of phosphorylation of ACC by AMPK on palmitoyl-CoA inhibition of ACC. This study was designed to determine if phosphorylation of muscle ACC by AMPK influences the extent of palmitoyl-CoA inhibition.

**MATERIALS AND METHODS**

*ACC Isolation.* Rats (Sprague-Dawley, Sasco, Charles River) were given free access to food and water and were kept in a room with a 12:12 light dark cycle. Rats were anesthetized with sodium pentobarbital (52 mg/100 g body weight). Quadriceps, hamstrings, and gastrocnemius muscles from 5-6 rats (Body weight 400-600 grams) were removed and placed between stainless steel blocks at ice temperature for quick cooling. Visible fat tissue was removed so as to only isolate skeletal muscle ACC. ACC was isolated as described previously (48). Muscles were placed in buffer (9 ml/gram muscle, wet weight) containing 225 mM mannitol, 75 mM sucrose, 10 mM tris-HCl, 0.05 mM EDTA, 5 mM potassium citrate, and 2.5 mM MnCl₂ at pH 7.5. Leupeptin (10 mg/liter),
antitrypsin (10 mg/liter) and aprotonin (10 ml/liter) were added immediately prior to use. To optimize collection of non-phosphorylated ACC, phosphatase inhibitors were not utilized in the homogenizing medium. Muscles were minced with scissors and then homogenized with a Brinkmann Polytron PT 3000 homogenizer. After centrifugation at 17,000 X g for 30 min at 4 °C, the supernatant was combined with ammonium sulfate granules (200 grams/liter) and stirred at 4 °C for one hour. After centrifugation (17,000 X g for 30 min at 4 °C) the supernatant was poured off and the precipitate was suspended in a minimal volume of buffer B (100 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 0.1 mM DTT, and 10% glycerol at pH 7.5). Proteolytic enzyme inhibitors were added at the same concentration indicated for buffer A. A homogenizer was utilized to resuspend the pellet. This resuspended solution was dialyzed for 3 hr against buffer C (100 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 0.1 mM DTT, 5% glycerol, pH 7.5). The dialyzed solution was centrifuged at 40,000 X g for 15 min at 4 °C to remove any insoluble material. The supernatant was applied to a freshly regenerated column of Promega Soft-Link, Soft-release avidin resin (Promega, Madison, WI). The column was washed with approximately 50 ml of buffer C. Elution buffer (100 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 0.1 mM DTT, 5 mM biotin, and 5% glycerol, pH 7.5) was then added to the column and allowed to stand at 4 °C for two hr. ACC was then eluted from the column with elution buffer at a rate of 2-3 ml/hr. Elutions fractions were analyzed for ACC activity. Active fractions were pooled and kept at -90 °C until utilized in assays. ACC activity from five different isolations was analyzed separately with respect to effect of phosphorylation on inhibition by palmitoyl-CoA.
**ACC Phosphorylation.** Globulin free albumin was added to aliquots of the ACC preparation to a concentration of 1 mg/ml. The presence of albumin greatly enhanced the yield of active enzyme collected in subsequent steps. ACC was then precipitated by addition of an equal volume of saturated ammonium sulfate solution. Samples were mixed, placed on ice 15 min and then centrifuged at 48,000 X g 15 min at 4 ºC. After discarding the supernatant, ACC was resuspended at 0.32 times the original volume in suspension buffer (68 mM HEPES, 126 mM NaCl, 13.6% glycerol, 1.36 mM EDTA, 1.36 mM EGTA, 1.36 mM DTT, pH 7.4). Final concentrations in the phosphorylation mix were 34 mM Hepes, 68 mM NaCl, 0.68 mM EDTA, 0.68 mM EGTA, 0.68 mM DTT, 6.8% glycerol, 0.12 mM ATP, 3 mM MgCl$_2$, 0.2 mM AMP, and 5 units/ml AMPK, pH 7.0. The AMPK, provided by Dr. D.Grahame Hardie, was purified from rat liver as described previously (48). In preliminary experiments, tubes were incubated for 0, 15, 30, and 47 min in a 30 ºC water bath to determine the incubation time required for maximal phosphorylation (determined by western blot, described below). For ACC used for activity assays, the 30 min incubation time was used routinely in the phosphorylation step of the procedure.

**Electrophoresis and Western Blots.** To verify phosphorylation, the incubation mixes from above were added to Laemmli’s solution and proteins separated on 5% SDS-PAGE gels (Tris-HCl Ready Gels, Bio-Rad, Hercules, CA) and then transferred to nitrocellulose membranes at 100 V for 50 min. Membranes were blocked in 5% dried milk (Bio-Rad) in PBST (139 mM NaCl, 2.7 mM KH$_2$PO$_4$, 9.9 mM Na$_2$HPO$_4$, and 0.1% Tween 20) for one hour. The membrane designated for phospho-ACC determination was left overnight with phospho-ACC polyclonal antibody (Cell Signaling Technology,
Beverly, MA) in 5% nonfat dried milk solution (1:5,000 dilution). Both membranes were washed twice in PBST and twice in PBS. The membrane designated for ACC determination was then exposed to streptavidin-horseradish peroxidase conjugate (Amerham Life Sciences, Arlington Heights, IL) in PBST (1:5,000 dilution) for 1 hr at room temperature. The membrane designated for phospho-ACC determination was exposed to horseradish peroxidase conjugated donkey anti-rabbit IgG (Amersham Life Sciences) in 3% nonfat dried milk in PBST (1:1,500 dilution) for 1 hr at room temperature. After being washed twice with PBST and twice with PBS, the membranes were incubated in enhanced chemoluminescence-detection reagent and then visualized on enhanced chemoluminescence hyperfilm (Amersham Life Sciences). Relative amounts of ACC and phospho-ACC were then quantified using SigmaGel software (SPSS, Chicago, IL).

**ACC Activity Measurements.** The ACC activity assay was performed on phosphorylated ACC and non-phosphorylated ACC. Final concentrations in the assay mix were 50 mM Hepes, pH 7.5, 1.5 mM MgSO$_4$, 2 mM DTT, 4 mM ATP, 12.5 mM KHCO$_3$, 2 μCi $[^{14}$C-bicarbonate], 20 mM magnesium acetate, 0.75 mg/ml fatty acid free bovine serum albumin with varying amounts of citrate, acetyl-CoA and palmitoyl-CoA in a final volume of 0.19 ml. In substrate (acetyl-CoA) activation curves citrate concentration was 20 mM and acetyl-CoA concentration varied with values at 0, 0.0025, 0.005, 0.010, 0.025, 0.050, 0.100, 0.250, and 0.500 mM and the palmitoyl-CoA concentration varied at 0, 0.001, 0.010 and 0.100 mM. For the citrate activation assay, citrate concentration was varied at concentrations of 0.00, 0.20, 0.50, 1.00, 2.50, 5.00, 10.00, and 20.00 mM, acetyl-CoA was held at 0.500 mM, and two palmitoyl-CoA
concentrations of 0.00 and 0.01 mM were used. The reaction was started by addition of 0.01 ml phosphorylated or non-phosphorylated ACC. After 4 min incubation at 37 ºC the reaction was stopped by addition of 50 µl 5 M HCl. After mixing and centrifugation 200 µl was added to a 7 ml scintillation vial and evaporated to dryness at 80 ºC. The residue (containing malonyl-CoA) was dissolved in 0.4 ml water and mixed with 5.5 ml Ecolite (ICN, Costa Mesa, CA) for determination of radioactivity. Preliminary experiments indicated linearity with time and enzyme concentration in this range. Sigma Plot 8.0 Software (Jandel Scientific) for Enzyme Kinetics was utilized for analysis of the data and preparation of curves of best fit.

Statistical Analysis. Statistical data for the family of inhibition or activity curves was obtained by the Sigma Plot 8.0 Software (Jandel Scientific) including standard error and R² correlation coefficient for Enzyme Kinetics or Number Crunching Statistical Software (NCSS) was used to perform analysis of variance and Fisher least significant difference test (for comparison of 3 or more means). The paired t-test was used for comparison of two means.

RESULTS

In order to determine the incubation time required for maximal phosphorylation of ACC by AMPK, western blot technique was used. Using a phospho-ACC antibody, membranes were probed to show the extent of phosphorylation as a function of time. As a control, duplicate blots were probed with streptavidin to show total ACC, which showed no change in ACC content as a function of time. The data from four
determinations are shown in Figure 1, a representative blot is also shown. From this analysis it was determined that an incubation time of 30 min would be sufficient for maximal phosphorylation of ACC by AMPK. Therefore this time was used for all subsequent phosphorylations of ACC by AMPK.

In Figure 2 there are two families of ACC activity curves which were generated by incubating purified skeletal muscle with varying acetyl-CoA concentrations and four palmitoyl-CoA concentrations. Each curve represents five runs, each one from a separate isolation of ACC. Using the Sigma Plot Enzyme Kinetic Software, the mixed partial form of inhibition gave the best fit analysis with an $R^2$ coefficient of 0.98. This implies both competitive and non-competitive components of inhibition by palmitoyl-CoA on ACC. As seen in the plots it is apparent that palmitoyl-CoA is a more effective inhibitor after ACC has been phosphorylated by AMPK.

Table 1 shows the values generated from the mixed partial inhibition analysis. $V_{\text{max}}$, $K_a$ for acetyl-CoA were unaffected by AMPK phosphorylation while the $K_i$ for palmitoyl-CoA was not only statistically different but also markedly reduced. This implies that when ACC is phosphorylated by AMPK less palmitoyl-CoA is needed for greater inhibition.

In Figure 3, $V_{\text{max}}$ as a function of acetyl-CoA concentration for the four different palmitoyl-CoA concentrations is shown. The reduction in the $V_{\text{max}}$ is proportionally lower in the phosphorylated ACC compared to non-phosphorylated. These differences were statistically significant for all of the palmitoyl-CoA concentrations ($p<0.05$).

Figure 4 shows ACC activity as a function of citrate concentration. It is shown for non-phosphorylated and AMPK phosphorylated ACC with or without the presence of
0.01 mM palmitoyl-CoA. As is apparent in these curves there is a marked decline in phosphorylated ACC activity in the presence of palmitoyl-CoA. In fact, statistically, only the curve with both phosphorylation and an inhibitor present is significantly different from the other three curves.

Figure 5 shows the effect of phosphorylation and palmitoyl-CoA at two specific citrate concentrations of 20 and 0.5 mM. The effect of palmitoyl-CoA inhibition is greater in the AMPK phosphorylated state than non-phosphorylated and this effect is even more exaggerated at the lower citrate level which was used to demonstrate physiological conditions. Statistically there was no difference at the high citrate level between the phosphorylated and non-phosphorylated without palmitoyl-CoA present but, there was a significant difference for all other phosphorylated and non-phosphorylated pairs.

DISCUSSION

For the results, CPM/min was used to show activity instead of the more standard protein concentration per rate. The reason why this was used was because of the inability to maintain the stability of the ACC enzyme as seen in activity. During storage of ACC after isolation the absolute activity would decrease in time. It was unfeasible to perform activity assays directly after isolation because of the enormous time constraints both processes required. As a result, activity would be lost even though protein concentration would remain the same. This would make the calculation of protein content inconsistent with activity. Therefore CPM/min was adopted as the form to be used for demonstrating activity instead of converting to concentration of protein. An activity assay would be run
on a sample right before the actual assay and concentration adjustments would be made from these results instead of using protein concentration for concentration adjustments.

The mixed partial form of inhibition was shown to have the highest correlation by having the highest $R^2$ coefficient. Not only does the mixed form of this inhibition infer competitive and non-competitive components but it is also conceivable the enzyme can produce product with the inhibitor attached. In fact competitive partial and uncompetitive partial models also gave $R^2$ coefficients above 0.9. This demonstrates a strong partial component suggesting as stated in the results that there exists a palmitoyl-CoA inhibition site away from the active site where palmitoyl-CoA is able to influence the activity of the enzyme. These data differ from the past data in which it was thought palmitoyl-CoA influenced ACC in a completely competitive inhibitory manner (41).

In looking at metabolic diseases such as obesity and diabetes the study of regulation of ACC has become more important. The recent formation of ACC-beta -/- null mutant mouse phenotype demonstrated why there needs to be an understanding of the natural regulatory mechanisms (2). These mice had less fat mass and higher rates of fatty acid oxidation, while continuing to be fertile and have a normal life span(2). Even when being fed high fat and high carbohydrate reduced weight gain and insulin-sensitivity were attenuated (1).

In looking at the above data, using the natural methods of inactivating ACC beta are plausible and important. Muscle contraction has been demonstrated to activate AMPK, which then phosphorylates and inactivates ACC beta (17,29,31,42,48). Contraction induced by electrical stimulation in rat muscle has been shown to decrease malonyl-CoA levels (31,33,46-48). This decline in malonyl-CoA has been postulated to
be important in allowing fat oxidation to proceed in the working muscle (44-46,49). In hindlimb perfusion studies, fatty acid oxidation increases when AMPK is chemically activated using AICAR (23,24,50). A good correlation was observed between the rate of palmitate oxidation and the malonyl-CoA content of muscle (23,24,50).

Palmitoyl-CoA has been reported to be an allosteric inhibitor of ACC isolated from skeletal muscle (41). Skeletal muscle is not considered to be a lipogenic tissue and the ACC beta is thought to be involved primarily in regulation of fatty acid oxidation. During fasting the mechanism of increased fatty acid oxidation to provide the energy needs of skeletal muscle is thought to be mediated through a decline in malonyl-CoA. During prolonged exercise, it is also important that malonyl-CoA content of the muscle be reduced to relieve inhibition of CPT1 and allow fatty acid oxidation to proceed to provide ATP for muscle contraction. This contraction-induced decrease in malonyl-CoA is thought to be mediated by AMPK phosphorylation/inactivation of ACC beta and by AMPK induced activation of malonyl-CoA decarboxylase (37,38,44,45,49). Results of the present study suggest that ACC phosphorylation not only produces direct covalent modulation/inactivation, but also enhances the ability of palmitoyl-CoA to inhibit ACC which reduces malonyl-CoA synthesis. The dual control insures that ACC will be inactivated, allowing fatty acid oxidation to proceed.

Clark and colleagues (12) demonstrated that elevated fatty acids (palmitate and oleate) can cause increased phosphorylation/activation of AMPK in perfused heart in the absence of changes of the energy status of the cell as seen in content of AMP, ADP, ATP and creatine phosphate. It is not known if this mechanism exists in skeletal muscle. It would seem possible that elevated fatty acids would have a mechanism to facilitate their
own oxidation by phosphorylation and inactivation of ACC. While Clark used palmitate and oleate to look at effects of AMPK and ACC activity, we only used palmitoyl-CoA as a representative of fatty acids in our inhibition of ACC. Similar results of palmitoyl-CoA activation of AMPK in liver were seen by Carling (11).

Fasting, high-fat diets, and exercise have been shown to increase rat skeletal muscle long chain fatty acyl-CoA (15,43). Values in the range of 1.6 to 8.3 µmol/Kg have been reported for rat muscle (15). A six fold increase of long chain acyl-CoA from 18 to 82 µmol/Kg dry mass has been demonstrated in humans during long term exercise (43). From this study the sensitivity range of muscle acetyl-CoA carboxylase thus appears to be in the range of fluctuation of long-chain acyl-CoA concentrations observed in muscle. It is also apparent that a phosphorylation-induced decrease in the Ki for palmitoyl-CoA inhibition of ACC would be expected to have physiologically relevant consequences in inhibiting malonyl-CoA synthesis.

High glucose levels are present in skeletal muscle in situations of insulin resistance and type 2 diabetes. Ruderman has demonstrated that by infusing rats with high glucose and insulin there were increases in the concentration of malonyl-CoA and citrate (21,39). These high glucose levels are part of a fuel-sensing mechanism proposed by Ruderman which lead to increases in citrate concentration that activates ACC, and to high malonyl-CoA levels thus reducing fatty acid oxidation (36). It is apparent from our data that the combination of AMPK phosphorylation with palmitoyl-CoA inhibits ACC in the presence of high and physiological citrate concentrations, therefore possibly shifting the fuel use of the cell when fatty acids are available and AMPK is activated.
Malonyl-CoA has previously been shown to be a competitive inhibitor for ACC in our lab by Trumble et al (41). In that study the $K_i$ for malonyl-CoA was found to be 10.6 ± 1.0 µM (41). We did not assay the effect of malonyl-CoA on AMPK phosphorylated ACC at this time. Future research in this area would provide valuable information on the effect of AMPK phosphorylation of ACC activity when malonyl-CoA levels are elevated. Elevated malonyl-CoA levels occur in insulin resistance. Ruderman specifically showed elevated malonyl-CoA levels in hyperglycemic and hyperinsulinemic rodents (39).

Malonyl-CoA concentration should be noted as only one mechanism controlling the rate of fatty acid oxidation in the muscle. Fatty acid concentration, availability of carnitine, concentration of acetyl-CoA (feedback inhibitor of fatty acid oxidation via the thiolase reaction), the availability of CoA in the mitochondrial matrix, and the overall energy charge all must be considered factors influencing total muscle fatty acid oxidation rate (19,45).

Another way to look at the data was to make inhibition curves for specific concentrations of acetyl-CoA. This is very similar to the apparent Vmax data, but it provides the opportunity to look at the lower concentrations of acetyl-CoA. This is advantageous for two of reasons; first this information is obscured in the activity curves. These graphs show the results and effectiveness of inhibition in lower acetyl-CoA concentrations. The second reason that this information is important is it allows us to look at the data in the physiological range of acetyl-CoA. This information is presented in figure 6 which shows the inhibition curves for 0.005mM and 0.01mM. The slope of the curve from the 0.0mM to 0.001 palmitoyl-CoA concentration is steeper for the non-phosphorylated curve compared to the phosphorylated curve. This demonstrates that at
low acetyl-CoA concentrations the AMPK phosphorylated ACC is already maximally inhibited and increasing amounts of palmitoyl-CoA are required to exhibit the same inhibitory influence. This not only demonstrates physiological importance but also shows that the effects of these two separate inhibitory methods are not merely additive but when combined produce and overall greater inhibition. The curves come together in the high Palmitoyl-CoA concentration. This is probably due to the low acetyl-CoA concentration and high palmitoyl-CoA concentrations.

In summary, palmitoyl-CoA was able to inhibit both non-phosphorylated and AMPK-phosphorylated ACC isolated and purified from rat skeletal muscle. The importance of palmitoyl-CoA as an inhibitor is seen by the dramatic decrease in Ki. Not only did AMPK phosphorylation have a direct effect but also increased the sensitivity of ACC to be inhibited by palmitoyl-CoA. There is also a decreased ability of citrate to activate AMPK phosphorylated ACC when palmitoyl-CoA is present. This is important during long-term exercise when AMPK is activated and there is elevated fatty acyl-CoA in the muscle. Therefore exercise is an effective natural means to cause inactivation of ACC beta, providing a method to prevent or reverse the effects of obesity and type 2 diabetes.
REFERENCES


Table 1. Effect of phosphorylation of ACC with AMP-Activated Protein Kinase on calculated Vmax, Km, and Ki

<table>
<thead>
<tr>
<th></th>
<th>Non-Phosphorylated</th>
<th>Phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vmax (cpm/min)</td>
<td>3158 ± 61</td>
<td>2768 ± 65</td>
</tr>
<tr>
<td>Km (acetyl-CoA) (µM)</td>
<td>45 ± 3</td>
<td>54 ± 4</td>
</tr>
<tr>
<td>Palmitoyl-CoA Ki (µM)</td>
<td>1.7 ± 0.25</td>
<td>0.85 ± 0.13*</td>
</tr>
</tbody>
</table>

*Significantly different from Non-Phosphorylated ACC, p < 0.01 (n = 5)
Figure 1. Western blots of Phospho-ACC (top blot) and ACC (bottom blot) at times following addition of AMPK buffer or buffer containing no AMPK to the reaction mix. The graph shows summary of mean data from 4 determinations (n = 4 at each point). Values are means +/- sem and are significantly different at 15, 30, and 45 min (p<0.001)
Figure 2. Substrate saturation curves of non-phosphorylated ACC and of AMPK-phosphorylated ACC at different concentrations of palmitoyl-CoA (n = 5 ACC activity assay each from a separate isolations at each point).
Figure 3. Effect of palmitoyl-CoA concentration on Vmax of non-phosphorylated ACC and of AMPK-phosphorylated ACC (n = 5 isolations of ACC which were used for non-phosphorylated and phosphorylated ACC at each palmitoyl-CoA concentration). * Significant difference from non-phosphorylated pair, p<0.05.
Figure 4. Citrate activation saturation curves of non-phosphorylated ACC and of AMPK-phosphorylated ACC at 0.0 mM and 0.01 mM concentrations of palmitoyl-CoA (n = 5 ACC activity runs all from the same isolation batch). * Significant difference from non-phosphorylated pair at same palmitoyl-CoA concentrations, p<0.05.
Figure 5. Activity of non-phosphorylated and AMPK phosphorylated ACC at 0.0 mM and 0.01 mM palmitoyl-CoA concentrations and at 20 mM and 0.5 mM citrate concentrations. (n=5 different runs under the same conditions from same ACC isolation batch for each point). * Significant difference from non-phosphorylated pair at the same citrate and palmitoyl-CoA concentrations, p<0.05.
Figure 6. Inhibition Curves. These are inhibition curves for two concentrations of Acetyl-CoA one at top 0.05mM and bottom 0.01mM against four palmitoyl-CoA concentration of 0.00, 0.10, 0.01 and 0.001mM. The curves show activity vs. palmitoyl-CoA concentration. Open circles are non-phosphorylatyed closed circle phosphorylated. Analysis of variance showed no significant difference but paired T-test for phosphorylated vs. non-phosphorylated at the same palmitoyl-CoA showed significant difference (p<0.05).