PKA as an Upstream Kinase for LKB1/STRAD/MO25

Seth Taylor Herway
Brigham Young University - Provo

Follow this and additional works at: https://scholarsarchive.byu.edu/etd

Part of the Cell and Developmental Biology Commons, and the Physiology Commons

BYU ScholarsArchive Citation
https://scholarsarchive.byu.edu/etd/933

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact scholarsarchive@byu.edu, ellen_amatangelo@byu.edu.
PKA AS AN UPSTREAM KINASE FOR LKB1/STRAD/MO25

by

Seth T. Herway

A thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Physiology and Developmental Biology

Brigham Young University

August 2006
This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory:

______________________    _______________________
Date       William W. Winder, Chair

______________________    _______________________
Date       James P. Porter

______________________    _______________________
Date       Craig D. Thulin
As chair of the candidate’s graduate committee, I have read the thesis of Seth T. Herway 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.

__________________________   ________________________
Date       William W. Winder
Chair, Graduate Committee

Accepted for the Department

__________________________
James P. Porter
Department Chair

Accepted for the College

__________________________
Rodney Brown
Dean, College of Biology and Agriculture
ABSTRACT

PKA AS AN UPSTREAM KINASE FOR LKB1/STRAD/MO25

Seth T. Herway
Department of Physiology and Developmental Biology
Doctor of Philosophy

The LKB1/STRAD/MO25 complex (LSMK) has been identified as the major upstream kinase for AMP-activated protein kinase (AMPK). PKA phosphorylates LKB1 at the Ser428 residue in humans and Ser431 residue in mice. We investigated PKA as an upstream kinase for LSKM. LKB1 that had been incubated with PKA prior to incubation with AMPK experienced up to a 51% increase in AMPK Kinase activity compared to LKB1 alone (p < 0.05). When blocked with a PKA Inhibitor, the kinase effect of PKA on LKB1 was eliminated. Rat epitrochlearis muscle tissue incubated with epinephrine experienced no increase in AMPK activity compared with controls indicating that epinephrine does not cause AMPK activity in this type of tissue. In conclusion, phosphorylation by PKA can increase the AMPKK activity of LKB1-STRAD-MO25 in vitro. Because LKB1 has been found to be constitutively active, it is postulated that phosphorylation by PKA may act to enhance LKB1-AMPK interaction and thus achieve its effect.
I thank Dr. William Winder for giving me the opportunity to work in a lab where quality research is being done and for helping me learn to become a scientist. I thank Dr. Eric Taylor for being there for me as I took my first steps into the world of the biological sciences. I thank my committee, the faculty, and others in the lab that have helped me complete this journey.
## TABLE OF CONTENTS

**PROSPECTUS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Proposed Experiments</td>
<td>7</td>
</tr>
<tr>
<td>Methods</td>
<td>9</td>
</tr>
<tr>
<td>References</td>
<td>12</td>
</tr>
</tbody>
</table>

**THESIS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>18</td>
</tr>
<tr>
<td>Introduction</td>
<td>19</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>22</td>
</tr>
<tr>
<td>Results</td>
<td>27</td>
</tr>
<tr>
<td>Discussion</td>
<td>29</td>
</tr>
<tr>
<td>References</td>
<td>33</td>
</tr>
<tr>
<td>Figure Legends</td>
<td>38</td>
</tr>
<tr>
<td>Figures</td>
<td>41</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>41</td>
</tr>
<tr>
<td>Figure 2</td>
<td>42</td>
</tr>
<tr>
<td>Figure 3</td>
<td>43</td>
</tr>
<tr>
<td>Figure 4</td>
<td>44</td>
</tr>
<tr>
<td>Figure 5</td>
<td>45</td>
</tr>
<tr>
<td>Figure 6</td>
<td>46</td>
</tr>
<tr>
<td>Figure 7</td>
<td>47</td>
</tr>
<tr>
<td>Figure 8</td>
<td>48</td>
</tr>
<tr>
<td>Figure 9</td>
<td>49</td>
</tr>
</tbody>
</table>
Prospectus

Introduction

The 5’-AMP-Activated protein kinase (AMPK) is a master metabolic regulator responsible for directing cellular responses to an energy challenge. AMPK is hypothesized to phosphorylate proteins involved in both fatty acid oxidation and glucose uptake. AMPK exerts these effects so as to generate ATP, while switching off biosynthetic pathways that consume ATP. In this manner it functions as an energy-sensing molecule that directs the body to a homeostatic energy condition. The roles of AMPK in glucose uptake and fatty acid oxidation, as well as other proposed roles in mitochondrial biogenesis and increased insulin sensitivity, have directed significant attention to discovering how activation of AMPK occurs within the body. This study will explore the role of Protein Kinase A (PKA) in the biochemical phosphorylation cascade that results in activation of AMPK.

Activation of AMPK

AMPK is composed of three subunits, denoted α, β, and γ. The α subunit is the catalytic subunit of the enzyme and the β and γ subunits are non-catalytic (1, 2, 3, 4). Each subunit has multiple isoforms; there exists two for the alpha subunit, two for the beta subunit, and three for the gamma subunit. When the alpha subunit is phosphorylated at the threonine 172 amino acid residue, the AMPK molecule is activated (5, 6, 7). The gamma subunit has four Cystathione Beta Synthase (CBS) domains that come together to form two AMP binding sites. Binding of the first AMP

1
allows for binding of the second which exposes the kinase domain on the alpha subunit, making it available for phosphorylation at threonine 172. AMPK is dephosphorylated and deactivated by Protein Phosphatase 2-A (PP2A). A higher AMP/ATP ratio makes AMPK a better substrate for phosphorylation by AMPK kinase (AMPKK) and a poorer substrate for phosphatases.

AMPKK and AMPK can also be activated artificially by use of the chemical 5-azidoimidazole-4-carboxamide-riboside (AICAR) (8, 9, 10, 11, 12, 13). AICAR was reported to be taken up by the cells and phosphorylated to form an AMP analog, ZMP, which then activated AMPKK and AMPK in the same manner as AMP.

AMPK is activated by muscle contraction (25), electrical stimulation in situ (27), and contraction of isolated muscles in vitro (28).

**Regulation of Fatty Acid Oxidation by AMPK**

AMPK exerts its effects of fatty acid oxidation by inactivating acetyl-CoA carboxylase (ACC) (14, 15, 16). Active AMPK phosphorylates ACC and thereby inhibits its activity. By inactivating ACC the amounts of malonyl-CoA in the cell are decreased because active ACC converts acetyl-CoA to malonyl-CoA. Malonyl-CoA inhibits carnitine-palmitoyl-acyl-transferase-1 (CPT-1) which is rate limiting for transport of fatty acids into the mitochondria for oxidation and use as fuel. Thus activation of AMPK results in less active ACC and therefore less inhibition by CPT-1 of fatty acids into the mitochondria, resulting in increased fatty acid oxidation.
Regulation of Glucose Uptake by AMPK

The insulin-sensitive glucose transporter, GLUT-4, resides in two locations in the muscle (17, 18, 19, 20). In the basal or low insulin state, the GLUT-4 transporters reside in microvesicles beneath the sarcolemma and T-tubule membranes. In response to insulin or contraction, these transporters are translocated to the membranes of the T tubules and sarcolemma, thus increasing the capacity for moving glucose into the muscle fiber. AMPK has been identified as the mediator of contraction-induced translocation of GLUT-4 from the microvesicle to the membrane (30, 31, 32, 33).

The Role of LKB1 as an AMPKK

In 2003 researchers at the University of Dundee determined that endogenous and recombinant complexes of LKB1, STRADalpha/beta, and MO25alpha/beta activate AMPK via phosphorylation of Thr172, thus functioning as an AMPK kinase (AMPKK) in this manner (21). LKB1 is a serine/threonine kinase that was discovered originally as the product of the gene mutated in Peutz-Jegher syndrome (22, 23). The researchers found that size-exclusion chromatography from rat liver extracts eluted AMPKK1 and AMPKK2. Blotting antibodies against LKB1, STRADalpha, and MO25 alpha revealed the activity of AMPKK2 correlated with the presence of LKB1, STRADalpha, and MO25alpha. There was also a weaker signal for LKB1, STRADalpha, and MO25alphas in AMPKK1. AMPKK activity was immunoprecipitated from both AMPKK1 and AMPKK2. The ability of the AMPKK1 and AMPKK2 fractions to activate AMPK was almost completely eliminated by
immunoprecipitation with anti-LKB1 antibody but not a control immunoglobulin. The researchers also showed that STRADalpha and MO25alpha are necessary to generate an active complex because the amount of active LKB1 was limited by the availability of STRADalpha and MO25 alpha. It has been determined that LKB1 has an absolute requirement for STRAD for catalytic activity and this activity is enhanced by binding to MO25 which is thought to act as a scaffolding protein (24).

**Characterization of LKB1 and its role as an AMPKK**

The role of LKB1 as an upstream AMPK kinase is not yet clearly defined. It has been shown that AMPKK activity increases with increasing exercise intensity and duration in humans (34). The increase in AMPKK activity was directly related to the increase in AMPKalpha1 activity but much less than that of the alpha2 isoform. Another study demonstrated that LKB1 in skeletal muscle was not activated by electrical stimulation induced contraction, AICAR, or phenformin (35). Identification of the exact mechanisms and regulation of AMPKK may shed further light on its role in the metabolic regulation that is mediated by AMPK.

**LKB1 as a Master Kinase**

Recently it was reported that the LKB1-MO25-STRAD complex phosphorylates eleven of the twelve AMPK-related kinases NUAK1, NUAK2, BRSK1, BRSK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, and MARK4, but not MELK (25). The function of many of these proteins are still unclear. Activity of many of these AMPK-related kinases resulted in a greater than fifty fold increase in activity
upon phosphorylation by LKB1. The authors suggest that LKB1 functions as a master kinase for AMPK and AMPK-related kinases.

### PKA and AMPK Actions in the Body

There are a number of physiologic actions in which increased cellular concentrations of PKA occur simultaneously with phosphorylation and activation of AMPK. In the liver, cellular PKA concentrations are elevated when glucagon and catecholamines are at higher levels in the body and insulin is depressed. Under these conditions glycogen synthesis is inhibited and glycogenolysis is stimulated. It is under the same conditions that higher levels of activated AMPK would be expected to be observed in the cell. The actions of glucagon on liver tissue may be analogous to what is observed in muscle tissue. One of the best characterized actions of glucagon is elevation of intracellular cAMP and subsequent activation of PKA (37). It is possible that high concentrations of PKA in muscle tissue may lead to activation of AMPK.

The inactivation of ACC, which is mediated by AMPK, is also induced by increased PKA levels due to the actions of glucagon. The parallel actions of both AMPK and PKA in the inactivation of ACC provides further reason to investigate possible correlations between the two proteins.

The beta-adrenergic signaling pathway represents a prime regulator of triglyceride breakdown, acting by the accumulation of cAMP and subsequent PKA-dependent phosphorylation of hormone-sensitive lipase (HSL). The activity of HSL is regulated by both phosphorylation and translocation to the lipid droplet (40). PKA phosphorylates HSL at Ser563, Ser659, and Ser660. Ser563 is suspected to be the key
residue phosphorylated for HSL activation. It has been reported that AMPK phosphorylates HSL at Ser565 in vitro without any direct effect on HSL activity, but this abolished the further phosphorylation of HSL by PKA at Ser563 (41). Research has been done to support the idea that B-adrenergic agents activate AMPK via an intermediary rise in cAMP (and therefore PKA) and that this serves to enhance the lipolytic rate as mediated by AMPK (37). This relationship between PKA and AMPK as seen in the effects of beta-adrenergic agents may also indicate a relationship between the two in muscle tissue.

PKA Regulates LKB1 Activity

PKA phosphorylates LKB1 at the serine 431 residue (38). LKB1 is a tumor suppressor protein that regulates cell proliferation and polarity (39). LKB1 terminates with a functional prenylation motif following Ser431 of Ala-Cys-Lys-Gln-Gln. It has been demonstrated that phosphorylation of LKB1 at Ser431, but not its farnesylation at Cys433, is very likely to be significant in the mediation of the ability of LKB1 to suppress cell growth (36). Mutation of Ser431 blocked the ability of LKB1 to suppress cell growth in a tumor line that does not normally express LKB1. Mutation of Cys433 had no such effect. Mutating Cys433 to alanine blocked membrane localization while phosphorylation of Ser431 by PKA had no localization effect.

Hypothesis

Just as PKA may mediate the tumor suppression activity of LKB1 by phosphorylation at Ser431, it may also serve to enhance its AMPKK activity in the
same manner. It is hypothesized that LKB1 phosphorylated by PKA at Ser431 would result in increased AMPKK activity of LKB1 and suggest a possible role for PKA as an AMPKK kinase (AMPKKK).

**Proposed Experiments**

1) The appropriate antibody for detecting phosphorylated LKB1 by PKA must first be determined. Varying concentrations of PKA will be incubated with an LKB1/MO25/STRAD preparation. The reaction will be stopped with Laemmli’s buffer after varying amounts of time. The reaction mixture will then be subjected to a Western Blot for determination of the degree of phosphorylation of LKB1 by PKA. Antibodies from Cell Signaling Technology for LKB1 (Ser428), Immunuquest for LKB1 (Ser431), and Santa Cruz Biotechnology for LKB1 (Ser431) will be tested and the most accurate and efficient antibody will be determined. These experiments will also aid in elucidating appropriate amounts of PKA and LKB1 to use in reaction mixtures.

2) Activity assays will be performed to determine the phosphorylative effects of PKA on the SAMS and AMARA peptides in order to ascertain whether activity assays can be used in conjunction with PKA or if the PKA will phosphorylate one of the peptides under consideration and obscure the results of all assays.

3) A series of two-step activity assays will be performed in which PKA is incubated with LKB1 sufficiently to phosphorylate the protein as determined by results of Experiment 1. Phosphorylated LKB1 will then be incubated with
AMPK and the degree of phosphorylation of AMPK will be determined. The concentrations of LKB1, PKA, and AMPK as well as the times of incubation will be varied in order to determine the requirements for full AMPK activation.

4) Western blots will then be performed to validate the results of Experiment 3.

5) The series of two-step assays conducted in Experiment 3 will be confirmed by assays using a PKA Inhibitor (Sigma Aldrich) to validate that the effects observed were occurring as a result of PKA and not another element in the reaction mixture.

6) PKA Inhibitor (Sigma Aldrich) will also be used in a two-step assay involving PKA and LKB1. PKA and LKB1 will be incubated to allow for phosphorylation of LKB1 by PKA. PKA Inhibitor will then be used to inhibit residual PKA and AMPK will then be added to the reaction mix. In this manner the interference of residual PKA in the phosphorylation of AMPK by LKB1 can be determined.

7) The efficacy of the PKA Inhibitor used in Experiment 6 and 7 will be validated with activity assays by determining phosphorylative effects of PKA with and without the inhibitor using KempTide (Upstate), a substrate for phosphorylation by PKA.

8) Rat epitrochlearis will be subjected to varying concentrations of AICAR, epinephrine and combinations thereof. Exposure to said compounds will increase the concentrations of PKA in the muscle. The tissue will then be homogenized and studied by western blot to determine the degree to which ACC, AMPK, and LKB1 were activated/inactivated due to this elevated
concentration of PKA in the muscle. Results of this experiment will be compared with the results of previous in vitro experiments.

9) Kemptide will be used with the rat epitrochlearis tissue in Experiment 8 to verify that there were increased levels of PKA in the tissue when exposed to epinephrine. The effect of AICAR on PKA tissue concentrations will also be measured.

10) An immunoprecipitation assay using G-Sepharose protein on the rat epitrochlearii will be performed in order to verify the AMPK activity in the muscle. This will then potentially be correlated with the hypothetical increases in PKA and LKB1 phosphorylation resulting from the treatments with AICAR and epinephrine.

Methods

Buffers. Dithiothreitol (DTT), AMP, ATP, [γ^{32}]ATP, 4-(2-Aminoethyl)-Benzenesulfonyl Fluoride (AEBSF), benzamidine, leupeptin, soybean trypsin inhibitor, and AMARA peptide (Zinsser, UK) will be added just prior to use when included. Tissue Homogenization Buffer: 50 mM Tris-HCl, 250 mM Mannitol, 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1mM EGTA, 1 % Triton X-100, with or without 10% glycerol v/v, 1 mM benzamidine, 1 µg/ml soybean trypsin inhibitor, 1 mM DTT, 0.2 mM AEBSF, pH 7.4 at 4°C. AMPKK Homogenization Buffer: As with Tissue Homogenization Buffer but with 100 µM leupeptin. AMPK Storage Buffer: 50 mM Tris-HCl, 250 mM Mannitol, 1 mM EDTA, 1mM EGTA, 1 mM DTT, 0.02% (w/v) Brij-35, 10% v/v glycerol, pH 7.4 at 4°C. Laemmli’s Buffer:
AMPKK Assay Buffer: 100 mM Hepes, 200 mM NaCl, 20% glycerol, 2 mM EDTA, 12.5 mM MgCl$_2$, 0.5 mM AMP, 0.5 mM ATP, 2.0 mM DTT, pH 7.0.

Phosphorylation Buffer: 40 mM Hepes, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl$_2$, 0.2 mM AMP, 0.2 mM ATP, 0.33 mM AMARA peptide, 0.133 µCi/µl [$\gamma^{32}$]ATP, pH 7.0. PBS: 140 mM NaCl, 2.7 mM KCl, 2.1 mM KH$_2$PO$_4$, 9.9 mM Na$_2$HPO$_4$, pH 7.3. PBST: PBS with 1% Tween-20 (Calbiochem).

**AMPKK Activity Assays.** Activation of AMPK will be measured by $^{32}$P incorporation from [$\gamma^{32}$]ATP into SAMS or AMARA peptide. Depending on the assay, PKA (2 µl) and/or LKB1 (2 µl) will be incubated with AMPKK Assay Buffer (4 µl) and rAMPK in Storage Buffer (4 µl) for varying periods. Phosphorylation Buffer (15 µl) will be added and the reaction will be stopped by spotting 1 sq cm pieces of P81 filter paper (Whatman; Tewksbury, MA) with the final reaction mixture (15 µl). With Kemptide or LKB1tide assays, the AMARA or SAMS peptide in the Phosphorylation Buffer will be replaced with 0.775 mM Kemptide or LKB1tide. Filter papers will be washed 6 x 90 sec in 100 ml 1% phosphoric acid, rinsed with acetone, dried, and counted for 1 minute in 3 ml Ecolite (ICN; Irvine, CA).

**PKA Abundance Assay.** To measure the abundance of PKA in rat epistropheus muscle tissue, the tissues will be diluted 1:9 (v/v) in Homogenization Buffer with 0.5 mM 3-isobutyl-1-methylxanthine for phosphodiesterase inhibition. Diluted tissue homogenates will be incubated with AMPKK Assay Buffer and Phosphorylation Buffer. The AMARA peptide in the Phosphorylation Buffer will be replaced with 0.775 mM Kemptide. The reaction will be stopped by spotting 1 sq cm pieces of P81 filter paper (Whatman; Tewksbury, MA) with the final reaction mixture (15 µl). Filter
papers will be washed 6 x 90 sec in 100 ml 1% phosphoric acid, rinsed with acetone, dried, and counted for 1 minute in 3 ml Ecolite (ICN; Irvine, CA).

**Western Blots.** Homogenates will be diluted in 4 x Laemmli’s buffer and water (1:1:2) and then separated by SDS-PAGE at 200 V for 35 minutes in 7.5% Tris-HCl, 50 µl well, Ready Gels (Bio-Rad Hercules, CA). Proteins will be transferred from the gels to nitrocellulose membranes at 100 V for 50 min. Membranes will then be blocked in PBST and 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) for 1h. Membranes will be incubated overnight at 4 C in PBST, 5% blocking milk, and first antibodies. After incubation with the first antibody, membranes will be washed twice with PBST for 10 minutes and twice with PBS for 5 minutes. Membranes will then be incubated for 1h at room temperature in PBST, 3% blocking milk, and a second antibody. Following incubation with the second antibody, membranes will be washed twice with PBST for 10 minutes and twice with PBS for 5 minutes. Membranes will then be covered with ECL Western Blotting Detection Reagent (Amersham Pharmacia), enclosed in plastic wrap, and visualized on Hyperfilm ECL High Performance Chemiluminescence film (Amersham Pharmacia). Relative amounts of protein will be quantified by measuring spot size and intensity with AlphEaseFC Software (Alpha Innotech; San Leandro, CA).
References


36. Sapkota GP, Agnieszka K, Lizcano JM, Lain S, Arthur JSC, Williams MR, Morrice N, Deak M, Alessi DR. Phosphorylation of the Protein Kinase Mutated in Peutz-Jeghers Cancer Syndrome, LKB1/STK11, at Ser431 by p90RSK and cAMP-


Abstract

The LKB1/STRAD/MO25 complex (LSMK) has been identified as the major upstream kinase for AMP-activated protein kinase (AMPK). PKA phosphorylates LKB1 at the Ser428 residue in humans and Ser431 residue in mice. We investigated PKA as an upstream kinase for LSMK. LKB1 that had been incubated with PKA prior to incubation with AMPK experienced up to a 51% increase in AMPK Kinase activity compared to LKB1 alone ($p < 0.05$). When blocked with a PKA Inhibitor, the kinase effect of PKA on LKB1 was eliminated. Rat epitrochlearis muscle tissue incubated with epinephrine experienced no increase in AMPK activity compared with controls indicating that epinephrine does not cause AMPK activity in this type of tissue. In conclusion, phosphorylation by PKA can increase the AMPKK activity of LKB1-STRAD-MO25 in vitro. Because LKB1 has been found to be constitutively active, it is postulated that phosphorylation by PKA may act to enhance LKB1-AMPK interaction and thus achieve its effect.

Key Words: AMPK, AMPKK, PKA, Epinephrine, LKB1
Thesis

Introduction

The 5’-AMP-Activated protein kinase (AMPK) is a master metabolic regulator responsible for directing cellular responses to energy challenges (9). AMPK phosphorylates acetyl CoA carboxylase (ACC) to promote fatty acid oxidation (21, 24, 26, 31) and other proteins to achieve insulin-independent glucose uptake (2, 6, 18). AMPK exerts these effects so as to generate ATP, while switching off biosynthetic pathways that consume ATP. In this manner it functions as an energy-sensing molecule that directs the body to a homeostatic energy condition. AMPK is activated by metformin, a commonly prescribed antidiabetic drug, which is thought to function by activating AMPK in the liver and muscle (36). The roles of AMPK in glucose uptake, fatty acid oxidation, and diabetic treatments, as well as other proposed roles in mitochondrial biogenesis and increased insulin sensitivity, have directed significant attention to discovering how activation of AMPK occurs within the body (9, 25, 27, 34, 37).

AMPK is phosphorylated and activated by an AMPK kinase (AMPKK). In 2003, a major AMPKK was identified to be LKB1 (STK 11) complexed with mouse protein 25 (MO25) and the STE-related adaptor protein (STRAD) (10). In muscle deficient in LKB1, phosphorylation of ACC-2 and glucose uptake were inhibited thus indicating a role for the LKB1 as an AMPKK in muscle (29). Additionally, mice lacking LKB1 expression in skeletal muscle had reduced AMPKalpha2 isoform activity as well as reduced phosphorylation of Thr172 (30).
LKB1 has been identified as the major AMPK kinase for skeletal muscle, but how it is regulated is not yet clearly defined. It has been shown that AMPKK activity increases with increasing exercise intensity and duration in humans (4). The increase in AMPKK activity was directly related to the increase in AMPKalpha1 activity but much less than that of the alpha2 isoform. Meanwhile, another study has demonstrated decreased AMPKK activity in a rat model with training duration and intensity despite increases in LKB1 protein (32). It has also been demonstrated that LKB1 in skeletal muscle was not activated by electrical stimulation induced contraction, AICAR, or phenformin (28). A study investigating AMPKK activity in trained or nontrained muscle in response to electrical stimulation showed that AMPKK activity did not increase even though phospho-AMPK did increase (14). This would suggest that AMPKK is activated during electrical stimulation of both trained and nontrained muscle by mechanisms other than covalent modification.

Identification of the exact mechanisms and regulation of AMPKK may shed further light on its role in the metabolic regulation that is mediated by AMPK.

LKB1 is a tumor suppressor protein that regulates cell proliferation and polarity (3). Mouse LKB1 is composed of 436 residues. LKB1 terminates with a functional prenylation motif following Ser431 of Ala-Cys-Lys-Gln-Gln. The human LKB1 protein is 433 residues long and terminates with the same residue sequence as in mouse tissue. In human tissue, the phosphorylation site is at serine 428. The catalytic domain is more distantly related to other protein kinases and its N-terminal and C-terminal non-catalytic domains are not related to any other proteins nor have
functional domains been detected therein (20). LKB1 has been found to be expressed in all fetal and adult tissues examined (12, 17).

There is evidence that LKB1 is phosphorylated at Ser31, Ser325, Thr 366, and Ser431 by upstream kinases. PKA phosphorylates LKB1 at Ser428 in humans and Ser431 in mice (30). LKB1 has been shown to autophosphorylate at Thr 185, Thr336, and Ser404 (1). It has been demonstrated that phosphorylation of LKB1 at Ser431, but not its farnesylation at Cys433, is very likely to be significant in the mediation of the ability of LKB1 to suppress cell growth (30). Mutation of Ser431 blocked the ability of LKB1 to suppress cell growth in a tumor line that does not normally express LKB1 (line G361). Mutation of Cys433 had no such effect. Mutating Cys433 to alanine blocked membrane localization while phosphorylation of Ser431 by PKA had no localization effect. Mutation of Thr336, the major autophosphorylation site, to a Glu, but not an Ala, prevented LKB1 from inhibiting cell growth in the G361 cell line (31). The Thr336, Thr366, and Ser431 residues as well as surrounding residues are highly conserved in mammalian LKB1 (31). The p90 ribosomal S6 protein kinase (RSK) and Protein Kinase A (PKA) phosphorylate LKB1 at the Ser431 residue (1, 5, 30). Therefore RSK and PKA may regulate cell growth by phosphorylation of LKB1. This regulation may extend to the AMPK kinase activity of LKB1.

The beta-adrenergic signaling pathway represents a prime regulator of triglyceride breakdown, acting by the accumulation of cAMP and subsequent PKA-dependent phosphorylation of hormone sensitive-lipase (HSL). The activity of HSL is regulated by both phosphorylation and translocation to the lipid droplet (13). PKA phosphorylates HSL at Ser563, Ser659, and Ser660. Ser563 is suspected to be the key
residue phosphorylated for HSL activation. It has been reported that AMPK phosphorylates HSL at Ser565 in vitro without any direct effect on HSL activity. Phosphorylation by AMPK abolished the further phosphorylation of HSL by PKA at Ser563 (7, 33). Research has been done to support the idea that beta-adrenergic agents activate AMPK via an intermediary rise in cAMP (and therefore PKA) and that this serves to enhance the lipolytic rate as mediated by AMPK (35). This relationship between PKA and AMPK as seen in the effects of beta-adrenergic agents may also indicate a relationship between the two in muscle tissue.

In the present paper, the effect of phosphorylation of LKB1 by PKA on the AMPK Kinase activity of LKB1 is reported. Additionally, we report the effect of increased PKA concentrations induced by exposure to the beta-adrenergic agent epinephrine on AMPK activation in rat epitrochlearis muscle tissue.

Materials and Methods

Animal care. All procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Male Wistar rats (Sasco, Wilmington, MA) were housed in a temperature controlled room (21-22 C) with a 12:12-h light-dark cycle. Rats were fed standard rat chow (Harlan Teklad rodent diet) and water ad libitum. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/Kg body wt).

Buffers. Dithiothreitol (DTT), AMP, ATP, [γ³²]ATP, 4-(2-Aminoethyl)-Benzenesulfonyl Fluoride (AEBSF), benzamidine, leupeptin, soybean trypsin inhibitor, SAMS peptide and AMARA peptide (Zinsser, UK) were added just prior to
use when included. *Kreb-Henseleit Buffer (KHB)*: 118.0 mM NaCl, 4.70 mM KCl, 
1.18 mM KH₂PO₄, 1.17 mM MgSO₄ (anhydrous), 25.0 mM NaHCO₃, 5 mM CaCl₂. 
Buffer oxygenated with 95% oxygen, 5%, carbon dioxide gas before use. *KHB-Gluc Buffer*: 2.8 mM ascorbic acid, 5 mM glucose, and 0.1 % BSA in oxygenated KHB. 
*KHB-BSA Buffer*: 1 mg BSA/ml KHB. *Insulin Stock Solution*: 1000 mU insulin in KHB-BSA Buffer. *Tissue Homogenization Buffer*: 50 mM Tris-HCl, 250 mM 
Mannitol, 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1mM EGTA, 1 % Triton X-100, with or without 10% glycerol v/v, 1 mM benzamidine, 1 µg/ml soybean trypsin inhibitor, 1 mM DTT, 0.2 mM AEBSF, pH 7.4 at 4°C. *Buffer C*: like muscle homogenization buffer but without NaF, sodium pyrophosphate, benzamidine, soybean trypsin inhibitor, and AEBSF. *AMPK Storage Buffer*: 50 mM Tris-HCl, 250 mM Mannitol, 1 mM EDTA, 1mM EGTA, 1 mM DTT, 0.02% (w/v) Brij-35, 10% v/v glycerol, pH 7.4 at 4°C. *Laemmli’s Buffer*: (30). *AMPKK Assay Buffer*: 100 mM Hepes, 200 mM NaCl, 20% glycerol, 2 mM EDTA, 12.5 mM MgCl₂, 0.5 mM AMP, 
0.5 mM ATP, 2.0 mM DTT, pH 7.0. *Phosphorylation Buffer*: 40 mM Hepes, 80 mM 
NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl₂, 0.2 mM AMP, 0.2 mM ATP, 1.0 mM SAMS peptide, 0.133 µCi/µl [γ³²]ATP, pH 7.0. 
*Immunoprecipitation Buffer*: 50 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 
ug/ml soybean trypsin inhibitor, pH 7.4. *Lysate Assay Buffer*: 62.5 mM Na HEPES, 
62.5 mM NaCl, 62.5 mM NaF, 6.25 mM pyrophosphate, 1.25 mM EDTA, 1.25 mM 
EGTA, 1mM DTT, 1mM benzamidine, 0.1 mM PMSF, 5 µg/ml SPTI. *PBS*: 140 mM 
NaCl, 2.7 mM KCl, 2.1 mM KH₂PO₄, 9.9 mM Na₃HPO₄, pH 7.3. *PBST*: PBS with 1%
Tween-20 (Calbiochem). *Immunoprecipitated AMPK Cocktail*: 0.1 ml of SAMS peptide was added to 0.20 ml of 100 mM Hepes, 200 mM NaCl, 20% glycerol, 2 mM EDTA, 12.5 mM MgCl2, 0.5 mM AMP, 2 mM DTT, 0.5 mM ATP, pH 7.0.

**Materials.** Reagents were obtained from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise stated. Recombinant LKB1-STRAD-MO25 lot #28144 (0.1 µg/µl, 34 units/mg) (AMPKK), LKB1tide, and Kemptide were obtained from Upstate (Charleston, VA). Anti-phospho Threonine-172 AMPK (P-AMPK) and anti-phospho Serine-431 LKB1 (P-LKB1) antibodies were obtained from Cell Signaling (Beverly, MA).

**Tissue Treatment.** Epitrochlearis muscle tissue (average weight 29.2 +/- 1.4 mg) was extracted from each rat and incubated for 45 minutes in one of six solutions immediately followed by clamp freezing and storage at -95 C. (1) The control group was incubated 100 µU insulin in KHB-Gluc Buffer. (2) The AICAR group was incubated in a solution of 2 mM AICAR and 100 µU insulin in KHB-Gluc Buffer. (3) The 1 uM Epi group was incubated in 100 µU insulin and 1 µM epinephrine in KHB-Gluc Buffer. (4) The 1 uM Epi + AICAR group was incubated in 100 µU insulin, 2 mM AICAR, and 1 µM epinephrine in KHB-Gluc Buffer. (5) The 25 nM Epi group was incubated in 100 µU insulin and 25 nM epinephrine in KHB-Gluc Buffer. (6) The 25 nM Epi + AICAR group was incubated in 100 µU insulin, 2 mM AICAR, and 25 nM epinephrine in KHB-Gluc Buffer.

**AMPKK Activity Assays.** AMPKK activity was measured by quantitating the increase in AMPK activity over time. The AMPK activity was measured by 

\[ ^{32}P \] incorporation from \([\gamma^{32}]ATP\) into SAMS peptide (10). LKB1 was incubated with
AMPKK Assay Buffer and rAMPK in Buffer C for 15 minutes. In assays determining the AMPKK activity of PKA, PKA was incubated with rLKB1 for 30 minutes prior to incubation with rAMPK and Buffer C for 15 minutes. All incubation times were determined by experimentally defining the kinetics of the reaction and by incubating for the period during which phosphorylation was linear as a function of time.

Phosphorylation Buffer was added to start the second step of the assay. Following a 10 minute incubation, the reaction was stopped by spotting 1 sq cm pieces of P81 filter paper (Whatman; Tewksbury, MA) with the final reaction mixture (15 µl). After waiting 20 sec for complete absorption, filter papers were placed in 1% phosphoric acid and then washed 6 x 90 sec in 100 ml of the acid, rinsed with acetone, dried, and counted for 1 minute in 3 ml Ecolite (ICN; Irvine, CA).

**PKA Phosphorylation Assay.** To determine the amount of phosphorylation by PKA and the efficacy of PKA inhibitor, Kemptide, a substrate for PKA phosphorylation, was used. Where Inhibitor was used, PKA in Buffer C was incubated with the Inhibitor in Buffer C for 30 min prior to beginning of assay. Phosphorylation Buffer with the 1.0 mM SAMS replaced with 0.775 mM Kemptide was then added to the mixture to start the reaction. Following a 20 minute incubation, the reaction was stopped by spotting 1 sq cm pieces of P81 filter paper (Whatman; Tewksbury, MA) with the final reaction mixture (15 µl). After waiting 20 sec for complete absorption, filter papers were placed in 1% phosphoric acid and then washed 6 x 90 sec in 100 ml of the acid, rinsed with acetone, dried, and counted for 1 minute in 3 ml Ecolite (ICN; Irvine, CA).
Western Blots. Homogenates were diluted in 4x Laemmli’s buffer and water (1:1:2) and then separated by SDS-PAGE at 200 V for 35 minutes in 7.5% Tris-HCl, 50 µl well, Ready Gels (Bio-Rad Hercules, CA). All blots that did not use tissue homogenates and involved incubation of proteins to achieve phosphorylation were stopped with 10 ul of 4x Laemmli’s buffer and then loaded into gels. Blots for pACC were done in 5% gels, respectively. Proteins were transferred from the gels to nitrocellulose membranes at 100 V for 50 min. Membranes were then blocked in PBST and 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) for 1h. Following blocking, membranes were incubated overnight at 4 C in PBST, 5% blocking milk, and first antibodies. After incubation with the first antibody, membranes were washed twice with PBST for 10 minutes and twice with PBS for 5 minutes. Membranes were then incubated for 1h at room temperature in PBST, 3% blocking milk, and a second antibody. Following incubation with the secondary antibody, membranes were washed twice with PBST for 10 minutes and twice with PBS for 5 minutes. Membranes were then be covered with ECL Western Blotting Detection Reagent (Amersham Pharmacia), enclosed in plastic wrap, and visualized on Hyperfilm ECL High Performance Chemiluminescence film (Amersham Pharmacia). Relative amounts of protein were quantified by measuring spot size and intensity with AlphEaseFC Software (Alpha Innotech; San Leandro, CA).

Immunoprecipitation. AMPK activity was determined by performance of immunoprecipitation as previously reported (22).

Statistics. A Student’s t-test was used to determine the effect on phosphorylation of AMPK upon incubation with LKB1 alone and incubation with LKB1 that has been
phosphorylated by 0.10 units of PKA as shown in Figure 3. All other experiments were analyzed for significance using a one-way analysis of variance (ANOVA). When effects were significant, the Fisher’s LSD multiple comparison test was used to determine the location. Statistical significance was set at p < 0.05 for all tests. All statistical procedures were performed with the NCSS statistical program (Kaysville, Utah). All data are reported as means +/- standard errors (SEM).

Results

To test the hypothesis that phosphorylation of LKB1 by PKA may increase the AMPKK activity of LKB1, we first incubated PKA with LKB1 and blotted for phosphoserine428-LKB1 (P-LKB1) to determine if LKB1 is phosphorylated by PKA at this residue. Two, 0.10, and 0.04 units of PKA as well as a buffer solution were incubated with LKB1. Maximal phosphorylation of LKB1 occurred with 0.10 phosphorylating units of PKA (Figure 1, n=6, p < 0.05).

To determine if LKB1 phosphorylated by PKA increases the AMPKK activity of LKB1, PKA (0.10 unit) was incubated with LKB1 as performed in the experiment shown in Figure 1. The phosphorylated LKB1 mixture was then incubated with AMPK and assayed using a SAMS peptide substrate to determine if additional phosphorylation of AMPK occurred. LKB1 that had been phosphorylated by 0.10 units of PKA prior to incubation with AMPK showed significantly more AMPKK activity than LKB1 alone (Figure 2, n=12, p < 0.05). The 0.10 unit mixture produced a 33% increase in phosphorylation compared to controls.
These results were then validated with a western blot for phosphothreonine-172 AMPK (P-AMPK) when LKB1 was incubated with AMPK having had prior incubation with 0.10 unit of PKA. Again, significant additional phosphorylation was seen with the mixture that had prior incubation with PKA as compared with LKB1 (Figure 3, n=6, p < 0.05). In the western blots, phosphorylation of AMPK was increased by 51% compared to LKB1 alone.

To confirm that the AMPKK Kinase effect of PKA was in fact due to the protein and not other elements in the reaction mixture, a series of experiments using PKA inhibitor were performed. First, Kemptide was used as a substrate for PKA phosphorylation to determine the efficacy of the inhibitor. PKA inhibitor effectively blocked the phosphorylation of Kemptide by PKA (Figure 4, n=6, p < 0.05). Then as in the experiments presented in Figure 2, an assay for AMPK activity was conducted using PKA as an AMPKKK for LKB1 compared with an assay wherein the PKA was previously incubated with the PKA inhibitor. PKA that had been incubated with an inhibitor showed no significant AMPKKK activity whereas PKA alone showed 19% more phosphorylation of AMPK than the mixture with the inhibitor and 25% more than LKB1 alone (Figure 5, n=6, p < 0.05).

To determine if the stimulatory effect of PKA on AMPKK activity could be demonstrated in intact muscle, rat epitrochlearis muscles were incubated without additions, with AICAR to activate AMPK, with epinephrine to activate PKA or with combinations of AICAR and epinephrine. As determined by immunoprecipitation, the groups treated with AICAR, 1 uM epinephrine, 1 uM epinephrine/AICAR, 25 nM epinephrine, and 25 nM epinephrine/AICAR all saw significant activation of AMPK
compared with controls (Figure 6, n=8, p < 0.05). The results of the AMPK activity assay were then verified with a blot for P-AMPK. Compared to controls, treatments with AICAR, 1 uM epinephrine/AICAR, and 25 nM epinephrine/AICAR all caused significant phosphorylation of the threonine-172 site on AMPK. The 1 uM and 25 nM epinephrine treatments had significant decreases in phosphorylation at the same site (Figure 8, n=6, p < 0.05). To corroborate the results of the blot for P-AMPK, a blot for phosphoserine-79 ACC (P-ACC) was performed. Significant phosphorylation of the site was seen in all the same treatments as in the blot for P-AMPK. However, there was no significant change in phosphorylation of serine-79 in the 1 uM and 25 nM epinephrine treatments (Figure 9, n=6, p < 0.05).

To verify that incubation with epinephrine resulted in increased amounts of PKA activity in the tissue, a blot for P-CREB, a downstream target of PKA was performed. All treatments except for the 25 nM epinephrine group caused increases in phosphorylation of the protein (Figure 9, n=6, p < 0.05).

**Discussion**

These results demonstrate that PKA can act as an AMPKK Kinase (AMPKKK) by means of its ability to phosphorylate serine residues on LKB1 thus stimulating its AMPKK activity. This was demonstrated both in assays and blots for phosphorylation of the threonine-172 site on the alpha subunit of AMPK. Increases of up to 51% in phosphorylation of this site due to prior phosphorylation of LKB1 by PKA were seen.
Incubating LKB1 with PKA showed the greatest effect on AMPK in activity assays, increasing activity by about 33%. Western blots using the same concentration of PKA resulted in an average increase in AMPK activation of nearly 51%.

It appears that the effect of PKA in increasing AMPK activity is due to its effect on LKB1 and not any direct action on the AMPK molecule. In the experiments shown in Figures 2 and 5, it can be seen that the same concentration of PKA that was incubated with LKB1 was also incubated with AMPK directly and no phosphorylation occurred. Additionally, experiments using PKA Inhibitor eliminated PKA’s ability to act as an AMPKKK.

The mechanism by which PKA may be increasing the AMPKK activity of LKB1 is unclear. LKB1 and calmodulin-dependent protein kinase kinase (CAMKK) are the only AMPK Kinases that have been identified although CAMKK’s role in skeletal muscle is not well-defined (11). Although the LKB1 complex may be regulated by upstream elements such as PKA, a number of studies suggest that the protein is constitutively active (8). Thus, it is likely that the kinase activity of the LKB1 complex may be regulated at the substrate level. Of the other 12 AMPK-related kinases, the LKB1/STRAD/MO25 complex phosphorylates and activates 11 of them (19). The LKB1 complex is not activated by contraction but contraction results in phosphorylation of AMPK giving evidence that the AMPKK activity of LKB1 is subject to target-specific control (13, 26).

Accordingly, it is possible that phosphorylation by PKA may not necessarily regulate the LKB1 complex itself but rather allow for better interaction between LKB1
and AMPK. It is postulated there is some kind of covalent modification induced by phosphorylation that begins to affect AMPKK activity.

Although there is a marked increase in phosphorylation of AMPK in vitro when LKB1 is previously phosphorylated by PKA, this effect was not seen in the epitrochlearis tissue. Phosphorylation of CREB, a downstream target of PKA, was utilized as an index of PKA activation. Of the five treatments to which the tissues were subjected, all but the tissues exposed to 25 nM epinephrine experienced significant increases in PKA activity. The fact that P-CREB increased in muscles exposed only to AICAR implies that AMPK may enhance activation of PKA in the absence of catecholamines. However, AMPK activation was only significant in tissues that had been treated with AICAR. Compared to the control, epinephrine alone did not result in significant activation of AMPK. The combination of AICAR and epinephrine also did not result in significant activation of AMPK any more than AICAR alone.

Whether used alone or in combination with AICAR, epinephrine did not result in increased AMPK activation in any of the tissues. The exception being noted in the immunoprecipitation experiment (Figure 6) wherein there was marginal activation of AMPK by 1 uM epinephrine that was just above the standard error. One study has shown stimulatory effects on AMPK in skeletal muscle cells mediated by $\alpha_1$-adrenoceptors, but not $\alpha_2$- or $\beta$-adrenoceptors (15). Another study has indicated that in brown adipocytes $\beta$-adrenoceptors, but not $\alpha$-adrenoceptors, stimulate AMPK (16). Additional work reports that the $\alpha$-adrenergic agonist, phenylephrine, stimulates $\alpha_2$-adrenoceptors. 
AMPK activity in soleus muscle tissue (22). The results of this study did not show an increase in AMPK activity as a result of incubation with epinephrine.

PKA’s ability to act as an AMPKKK as witnessed *in vitro* may not have been seen in the muscle tissue because the PKA is not regulating the LKB1 molecule itself but rather involved in enhancing the LKB1-AMPK interaction. Resultant cellular concentrations of PKA due to the tissue treatments may have been outside the ranges that resulted in the stimulatory effect on the AMPKK activity of LKB1 seen *in vitro*.

In conclusion, we found that phosphorylation by PKA can increase the AMPKK activity of LKB1-STRAD-MO25 *in vitro*. This effect was witnessed within specific concentration ranges of PKA. The stimulatory effect of PKA phosphorylation of LKB1-STRAD-MO25 was not observed in intact incubated muscle stimulated with AICAR and epinephrine. It is postulated that phosphorylation by PKA may act to enhance LKB1-AMPK interaction and thus achieve its effect.
References


16. **Hutchinson DS, Chernogubova E, Dallner OS, Cannon B, and Bengtsson T.** Beta-adrenoceptors, but not alpha-adrenoceptors, stimulate AMP-activated protein


31. **Sapkota GP, Boudeau J, Deak M, Kieloch A, Morrice N, Alessi DR.** Identification and characterization of four novel phosphorylation sites on
LKB1/STK11, the protein kinase mutated in Peutz-Jeghers cancer syndrome.


Figure Legends

Figure 1
Phosphorylation of LKB1 by PKA was verified by western blot showing relative quantities of phosphoserine-428 (P-LKB1) after incubation of PKA with LKB1. Optimal phosphorylation was determined to occur with 0.10 phosphorylating units of PKA. The buffer value is normalized to 1 (n=6, p < 0.05, * = significantly more than buffer).

Figure 2
Significant additional AMPK phosphorylation occurs when 0.10 units of PKA is incubated with LKB1 prior to incubation with AMPK as compared to when AMPK is incubated with LKB1 alone (n=12, p < 0.05, * = significantly more than LKB1 alone).

Figure 3
The results shown in Figure 2 are validated by western blot showing relative quantities of phosphothreonine-172 AMPK (P-AMPK) after incubation with LKB1 and incubation with LKB1 that has been phosphorylated by 0.10 units of PKA. Additional significant phosphorylation of the AMPK molecule is achieved when LKB1 is incubated with 0.10 units of PKA prior to incubation with AMPK (n=6, p < 0.05, * = significantly more than LKB1 alone).

Figure 4
As determined by assay using the substrate Kemptide, it was found that Kemptide is not phosphorylated by PKA when PKA is incubated with PKA Inhibitor prior to incubation with Kemptide (n=6, p < 0.05, * = significantly more than Inhibitor alone).

**Figure 5**

As determined by assay using SAMS peptide, incubation of PKA with PKA Inhibitor prior to incubation with LKB1 eliminates the additional activation of AMPK seen in the experiment shown in Figures 2 and 3. Thus it is shown that the effect is due to PKA and not other elements in the reaction mixture (n=6, p < 0.05, * = significantly more than LKB1 alone, + = significantly less than LKB1 alone).

**Figure 6**

Immunoprecipitation to determine AMPK activity in rat epitrochlearis muscle tissue. The AMPK activity of 1 uM Epinephrine + AICAR and 25 nM Epinephrine + AICAR compared with AICAR alone is not statistically significant (n=8, p < 0.05, * = significantly more than control).

**Figure 7**

Activation of AMPK was measured by western blot showing relative quantities of phosphothreonine-172 AMPK (P-AMPK). Significant phosphorylation occurred in the AICAR, 1 uM Epinephrine + AICAR, and 25 nM Epinephrine + AICAR treatments. Phosphorylation was significantly less than control in the 1 uM
Epinephrine and 25 nM Epinephrine groups (n=6, p < 0.05, * = significantly more than control, + = significantly less than control).

**Figure 8**
Phosphorylation of Acetyl CoA Carboxylase was measured by western blot showing relative quantities of phosphoserine-79 ACC (P-ACC). Significant phosphorylation occurred only in the AICAR, 1 uM Epinephrine + AICAR, and 25 nM Epinephrine + AICAR treatments (n=6, p < 0.05, * = significantly more than control).

**Figure 9**
Phosphorylation of CREB was determined by western blot for relative quantities of phospho-CREB. Significant phosphorylation occurred in all treatments except for the 25 nM Epinephrine group (n=6, p < 0.05, * = significantly more than control).
Figure 1
Figure 2

![Graph showing AMPK Activity (pmol/min/mg) for PKA/LKB1, PKA, and LKB1. The graph indicates a significant difference (*) in AMPK Activity between PKA/LKB1 and PKA.]
Figure 3

PKA/LKB1  LKB1  PKA/LKB1  LKB1

P-AMPK (Relative Units)

PKA/LKB1  LKB1

P-AMPK (Relative Units)
Figure 4

The diagram shows the levels of Phosphorylated Kemptide in different conditions:
- PKA
- PKA/Inhib
- Inhib

The y-axis represents the concentration of Phosphorylated Kemptide in pmol/min/mg, ranging from 0.00 to 0.10. The x-axis lists the conditions. The bar for PKA is significantly higher than the other conditions, indicated by an asterisk (*) above the bar.
Figure 5

AMPK Activity (pmol/min/mg)
Figure 6

AMPK Activity (pmol/min/ng)

Control  AICAR  1uM Epi  1uM Epi + AICAR  25nM Epi  25nM Epi + AICAR
Figure 8

Control  AICAR  1uM Epi  1uM Epi/A  25nM Epi  25nM Epi/A

P-ACC (Relative Units)

Control  AICAR  1uM Epi  1uM Epi + AICAR  25nM Epi  25nM Epi + AICAR
Figure 9

P-CREB (Relative Density)

Control  AICAR  1uM Epi  1uM Epi + AICAR  25nM Epi  25nM Epi + AICAR

*
Seth T. Herway

Current Address: 733 N. 600 E.  
Provo, UT 84606  
(801)362-3106  
Permanent Address: 20 Sijsjeslaan  
B-3080 Tervuren  
seth_herway@yahoo.com

Education
Brigham Young University  
Provo, UT  
B.S. in Chemical Engineering April 2003  
M.S. in Physiology and Developmental Biology April 2006  
GPA: 3.62/4.00

Experience
June 2003-  
ExxonMobil Development Company  
Houston, TX  
July 2004  
Project Controls Engineer  
• Managed contractor’s progress, cost, and schedule in projects in Chad and offshore Angola.  
• Created investment curves for all developing produced water treatment technologies.

April 2002-  
BYU Office of Technology Transfer  
Provo, UT  
April 2003  
Licensing Liaison  
• Discover and cultivate markets for new technologies in the fields of drug delivery, cell growth, instrumentation, and composite materials. Negotiate licensing agreements and assist in obtaining patents for new technologies.

Jan-Feb.  
Japan Broadcasting Corporation  
Salt Lake City, UT  
2002  
Broadcast Translator  
• Provided English/Japanese translation for interviews and live 2002 Winter Olympic broadcasts.

August-Dec.  
BYU Chemical Engineering Department  
Provo, UT  
2001  
Unit Operations Lab Teaching Assistant  
• Instructed students in lab procedures and principles of fluid mechanics, thermodynamics, and heat transfer.

June-August  
Huntsman International  
Brussels, Belgium  
2001  
Environmental Research Assistant-Polyurethanes  
• Conducted market analysis of potential processes for the conversion of byproduct HCl to Cl₂.  
• Identified process with a potential capital cost savings of $5 million and a $20 savings per ton of product.

July 1998-  
The Church of Jesus Christ of Latter-Day Saints  
Fukuoka, Japan  
July 2000  
Volunteer  
• Trained and supervised 150 other volunteers as Assistant to the President.  
• Conducted presentations for individuals and groups.  
• Developed fluency in the Japanese language.

Activities
American Institute of Chemical Engineers-Editor of Chapter Newsletter  
Brigham Young University Rugby Team  
Services for Students with Disabilities Volunteer  
Junior Achievement Volunteer  
United Way of the Texas Gulf Coast Advertising Committee

Honors
Tom and Della Hanks Scholar  
BYU Heritage Scholar  
Published in American Journal of Physiology-Endocrinology and Metabolism