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REGULATION OF LKB1-STRAD-MO25 COMPLEX EXPRESSION AND ACTIVATION OF AMPK IN SKELETAL MUSCLE BY THYROID HORMONE

by

Devon J. Branvold

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 2007

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

Devon J. Branvold

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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BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the thesis of Devon J. Branvold 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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ABSTRACT

REGULATION OF LKB1-STRAD-MO25 COMPLEX EXPRESSION AND ACTIVATION OF AMPK IN SKELETAL MUSCLE BY THYROID HORMONE

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Department of Physiology and Developmental Biology

Master of Science

AMP-activated protein kinase (AMPK), a heterotrimeric protein which serves as a metabolic master switch in skeletal muscle, is a research target for the pharmaceutical treatment and prevention of type 2 diabetes. The expression of all of the isoforms of the subunits of AMPK and AMPK activity are increased in skeletal muscle tissue of hyperthyroid rats. Activity of AMPK is regulated by an upstream kinase (AMPKK). The LKB1-STRAD-MO25 complex is a major AMPKK in skeletal muscle. This experiment was designed to determine whether the increase in AMPK activity is accompanied by a thyroid hormone-induced increase in the expression of the LKB1-STRAD-MO25 complex. LKB1-STRAD-MO25 complex protein expression was determined by Western blots in control rats, in rats given 3 mg of thyroxine and 1 mg of triiodothyronine per kilogram chow for 4 weeks, and in rats given 0.01% propylthiouracil (PTU) in drinking water for 4 weeks. The relative expression of LKB1, MO25, and STRAD, as well as PGC-1α, increased in the soleus of thyroid hormone treated rats vs. the controls. MO25 mRNA increased with thyroid hormone treatment, and STRAD mRNA increased with

PTU treatment. Phospho-AMPK and phospho-ACC increased in response to electrical stimulation in muscles of all treatment groups, but was most markedly increased in hyperthyroid rats. Thyroid hormone treatment also increased the amount of phospho-CREB in the soleus, heart, and red quadriceps. These data provide evidence that thyroid hormone partially controls expression of the LKB1-STRAD-MO25 complex, as well the subsequent activation of AMPK.

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TABLE OF CONTENTS

PROSPECTUS

Introduction	1
Proposal	10
Anticipated Experiments and Methods	12
References	19

THESIS

Abstract	29
Introduction	30
Materials and Methods	33
Results	36
Discussion	39
References	46
Figure Legends	54
Figures	57

List of Figures

Figure 1 57
Figure 2 59
Figure 3 60
Figure 4 61
Figure 5 62
Figure 6 64

List of Tables

Table 1 66

PROSPECTUS

Introduction

AMP-activated protein kinase (AMPK), a heterotrimeric protein which serves as a metabolic master switch in skeletal muscle, is a research target for the pharmaceutical treatment and prevention of type 2 diabetes. AMPK has been shown to have involvement in fatty acid oxidation, glucose uptake, and increase of citric acid cycle and electron transport chain enzymes. Many of the effects of thyroid hormone in skeletal muscle are similar, including increases in electron transport chain and citric acid cycle enzymes, enzymes involved in ketone oxidation, hexokinase and GLUT-4. In 2002 it was shown that treatment with thyroid hormone results in increased expression of all of the isoforms of the subunits of AMPK (1). AMPK activity was also shown to increase in the skeletal muscle tissue of thyroid hormone-treated rats. Activity of AMPK is regulated by an upstream AMPK kinase (AMPKK). The LKB1-STRAD-MO25 complex has been shown to be a major AMPKK in skeletal muscle. This experiment was designed to determine whether the increase in AMPK activity is due to a thyroid hormone-induced increase in the expression of the LKB1-STRAD-MO25 complex.

About 5'-AMP-activated protein kinase.

5'-AMP-activated protein kinase (AMPK) acts as a fuel gauge for the cell, responding to changes in cellular energy (2). When it "senses" low ATP, it turns off ATP-depleting pathways and switches on mechanisms of ATP production (2). AMPK is a heterotrimer consisting of a 63-kD alpha subunit, a 40-kDbeta subunit, and a 38-kD

gamma subunit (3). There are at least two isoforms of the alpha and beta subunits, and three isoforms of the gamma subunit.

The alpha subunit contains the site of phosphorylation by AMPKK, as well as the catalytic site for kinase activity (4, 5). The two alpha subunits differ somewhat in expression and function. Alpha1 is widely expressed throughout the body, whereas alpha2 expression is highly concentrated in cardiac and skeletal muscle and in the liver (4, 6). Alpha2 is stimulated to a much higher extent than alpha1 by 5'-AMP, and reactivation of the AMPK complex by AMPKK is stimulated by AMP to a much larger extent for the alpha2 complex (4). Both are activated by the same upstream kinase, and inactivated by PP2C and PP2A, but alpha2 is dephosphorylated much more readily by PP2A (4). Alpha1 is mainly cytosolic, whereas alpha2, although also expressed in the cytosol, is highly expressed throughout the nucleus, except in the nucleolus (4).

The beta subunit contains a highly conserved ASC domain used for protein binding (7). Some evidence shows that the beta subunit acts as a scaffold between the alpha and gamma subunits, but a recent study provided evidence that the beta and gamma subunits both bind to the alpha subunits, but not to each other (8). The beta1 subunit is predominantly expressed in the liver, but in skeletal muscle, beta1 and beta2 are expressed in fast twitch muscles and beta1 only is expressed in slow twitch muscles (9). The beta subunit contains a glycogen binding domain, and it seems that this association gives AMPK access to glycogen synthase and glycogen phosphatase, two enzymes with which AMPK co-immunoprecipitates (10).

The gamma subunit has three isoforms, which differ significantly in expression (11). The gamma1 subunit is much more highly expressed than the other two

in most tissues, except in the brain and testis, where all three seem to have nearly equal expression (11). Both gamma1 and gamma2 subunits are expressed in various tissues, whereas gamma3 seems restricted to skeletal muscle (11). All three readily complex with the alpha subunit without preference, but gamma1 is more often formed because it is much more prevalent (11). The gamma2 subunit is significantly more AMP-dependent than the other two isoforms (11). The gamma subunit contains four repeats of a protein module called cystathionine beta-synthase (CBS), which have a regulatory function (12). AMP binds to a set of two CBS domains (13), also called a "Bateman domain (14)." One model suggests that AMP binds to the CBS domain of the gamma subunit, stabilizing interaction between the alpha and gamma subunit, which opens up the autoinhibitory region of the alpha subunit, allowing the Thr172 site to be exposed and phosphorylated (11).

AMPK Activation

AMPK is activated during times of cellular stress, such as exercise, ischemia, and hypoxia (15). AMPK senses increases in the AMP/ATP ratio and the creatine/creatine phosphate ratio (15). AMPK is allosterically activated by AMP, and inhibited by CP and ATP (16), which allows for its sensitivity to cellular energy. ATP inhibits AMPK by binding to an allosteric site (5). AMP is reported to activate AMPK via three distinct pathways. The first pathway involves allosteric activation of AMP via binding to the gamma subunit (5, 16). AMP, when bound to the gamma subunit, also makes AMPK a poor substrate for protein phosphatase 2C (16, 17). The third pathway, that AMP-binding makes AMPK a better substrate for its upstream kinase, AMPKK, is currently in

dispute. Hardie *et al* (5), using partially purified AMPKK, reported that AMP allowed for increased phosphorylation of AMPK by its kinase. Carling *et al* (17, 18) reported that when using purified LKB1, the catalytic protein in the LKB1-STRAD-MO25 complex, a known AMPKK, AMP does not directly activate LKB1. AMP, also reportedly, does not promote phosphorylation of AMPK by CAMKK beta, another known AMPKK (17, 19, 20). Carling proposes that the results would be the same for LKB1, since both phosphorylate the same residue on AMPK (17). Carling suggests that protein phosphatase 2C in the imperfectly purified AMPKK is responsible for the results Hardie's lab observed (17). Whether Hardie's partially purified AMPKK or Suter's and Carling's recombinant LKB1 and AMPK, purified from bacteria (18), are indicative of what really occurs in mammalian cells will have to be delineated by further research.

In 2003, LKB1, STRAD, and MO25 were shown to be an upstream kinase for AMPK, with LKB1 in possession of the catalytic domain (21, 22). LKB1 is a serine/threonine kinase, which has been shown to be a tumor suppressor (21, 23). Mutations in LKB1 are linked to Peutz-Jeghers syndrome, in which multiple gastrointestinal polyps form and the likelihood of malignant tumors is greatly increased (24). LKB1 alone is mainly localized in the nucleus, but when associated with STRAD and MO25, it is anchored in the cytoplasm (21). STRAD and MO25 have no catalytic function (21). Association between LKB1 and STRAD is enhanced by increasing levels of MO25 in a dose-dependent fashion. Optimal activation of AMPK by LKB1 requires the presence of all three proteins (21, 25).

For over a decade it has been known that calmodulin-dependent protein kinase kinase-beta (CaMKKbeta) can phosphorylate and thereby activate AMPK, but it was not

the main AMPKK in liver (26). Richter *et al* (27) found that CaMKK inhibitors strongly inhibited AMPK phosphorylation in mouse soleus and EDL muscles after 2 minutes of contraction, but much less as time of contraction increased. CaMKK inhibitors had no effect on 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) phosphorylation and activation of AMPK (27). AICAR is taken into the cell and converted to ZMP, an AMP analog that has been shown to activate AMPK (28). Recent LKB1 knockout studies have shown that without LKB1, electrical and AICAR stimulation of muscle results in very little phosphorylation of AMPK and of ACC, providing evidence that LKB1-STRAD-MO25 is the major AMPKK in muscle (29).

AMPK and Lipid Metabolism

One of the effects of exercise is an increase in fatty acid metabolism, which provides more energy for the cell. One of the key pathways in AMPK's regulation of fatty acid oxidation is the phosphorylation and inactivation of acetyl-CoA carboxylase (30). Acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA, an inhibitor of carnitine parmitoyltransferase 1 (CPT-1). CPT-1 transports fatty acids into the mitochondria for oxidation. Inactivation of ACC, therefore, results in increased fatty acid transport and subsequent oxidation. It is also thought that the decrease in malonyl-CoA occurs as a result of malonyl-CoA decarboxylase (MCD), which may be regulated by AMPK (31). MCD is an antagonist to ACC, decarboxylating malonyl-CoA to acetyl-CoA, resulting in decreased malonyl-CoA and increased CPT-1 and fatty acid oxidation.

AMPK also plays an important role in lipid metabolism in the liver. It has long been known that hepatic ACC has been regulated in the liver by phosphorylation (32).

AMPK also phosphorylates and inactivates 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), a key enzyme in cholesterol synthesis (28). HMGR converts 3-hydroxy-3-methylglutaryl-CoA, which is made from acetyl-CoA, into mevalonic acid, which then travels down several more metabolic steps to become cholesterol. AMPK, therefore, helps regulate fatty acid oxidation and cholesterol synthesis.

AMPK and Glucose Transport

Insulin is a hormone which helps regulate glucose levels in the body. When blood glucose is high, insulin is released from the Islets of Langerhans. Insulin, among other things, will then facilitate the uptake of glucose into cells via increased expression and translocation of glucose transporter GLUT-4 (34). Under conditions of exercise, however, blood sugar levels are not necessarily high, and insulin concentration decreases, yet muscles are still able to bring in glucose. AMPK seems to be responsible in part for this exercise-induced glucose uptake. Goodyear *et al.* (33) observed that with exercise, the concentration of GLUT-4 was increased in the plasma membrane, but decreased in the microsomal membranes, suggesting that exercise facilitates the translocation of vesicular GLUT-4 to the plasma membrane. While acute exercise increases GLUT-4 translocation, endurance training will increase the total amount of GLUT-4 protein available (35). It has been shown that both electrical stimulation and AICAR treatment increase AMPK activation, glucose uptake, and GLUT-4 translocation in perfused rat hindlimb muscle, linking exercise-induced glucose uptake to AMPK (36, 37, 38). Chronic AICAR injections, simulating some of the effects of endurance training, also increase the total amount of GLUT-4 protein in the muscle cell (39).

Two proteins are essential for the regulation of GLUT-4 expression at a transcriptional level – myocyte enhancer factor 2 (MEF2) and GLUT-4 enhancer factor (GEF). Mutations in the DNA binding regions for either of these proteins results in ablation of transgene GLUT-4 expression (40, 41). These results prompted a study in 2005 which showed that AMPK directly phosphorylates GEF, but it doesn't seem to directly activate MEF2 (42). AICAR treatment has been shown, however, to increase transport of both proteins into the nucleus, as well as increase the binding of both to the GLUT-4 promoter region (42).

There is another protein involved in carbohydrate metabolism that is worthy of mention along with GLUT-4. The enzyme hexokinase phosphorylates a six-carbon sugar, most notably glucose, which is the first step in glycolysis. When glucose is transported into the cell it is phosphorylated by hexokinase. This phosphorylation keeps glucose from leaving the cell, and by changing the structure of glucose through phosphorylation, it decreases the concentration of glucose molecules, allowing a gradient for more glucose to be transported into the cell. Hexokinase II transcription is increased in both red and white skeletal muscle upon treatment with AICAR (43). With chronic injections of AICAR, total protein content of hexokinase II increases in rat skeletal muscle (44).

AMPK and Mitochondria

Mitochondria are often called the powerhouse of the cell. After pyruvate is formed from glucose during glycolysis in the cytoplasm, it is transported into the mitochondria, where it is oxidized to acetyl-CoA and enters the citric acid cycle.

Oxidative phosphorylation is the process by which NADH and $FADH_2$ are oxidized and oxygen is reduced. This process creates a proton gradient which is used to drive ATP synthase and produce ATP. This process creates energy for cellular properties, and since ATP is indispensable in the contraction process, so then, are mitochondria.

Mitochondrial enzymes, such as cytochrome c, succinate dehydrogenase, malate dehydrogenase, α-ketoglutarate dehydrogenase, and citrate synthase, increase in expression and activity in response to exercise (45). AICAR stimulation of AMPK increases cytochrome c and δ-aminolevulinate synthase (ALAS), a rate-limiting enzyme involved in the production of heme. Malate dehydrogenase and succinate dehydrogenase also increase, as well as citrate synthase activity, in rats treated with AICAR injections (46). Conversely, in LKB1 knockout mice, there are decreases in cytochrome c and citrate synthase activity, even if the mice are "trained" by voluntary exercise (47).

Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) is a transcriptional regulator for genes involved in mitochondrial biogenesis, fatty acid oxidation, and gluconeogenesis (48). To do this, it enhances the activity of transcription factors like nuclear respiratory factor 1 (NRF-1), myocyte enhancer factor 2 (MEF2), host cell factor (HCF), and others (49, 50). It also has a positive feedback loop, enhancing its own expression (51). Both MEF2 and cAMP response element (CRE) are essential for contraction-induced PGC-1α promoter activity (50). AMPK is required for increased PGC-1α expression in skeletal muscle in response to creatine depletion (52). LKB1 knockout mice show a decrease in PGC-1α, as well as mitochondrial proteins (47).

Thyroid Hormone

Thyroid hormone is a tyrosine derivative synthesized in the thyroid. There are two endogenous active forms of thyroid hormone, T_3 and T_4, and although T_3 is the more enzymatically active, T_4 is the more prevalent in the body. T_4 serves as a sort of prohormone since it has a much longer half life in the blood, and it is readily converted to T_3 by deiodinases in the tissue. Thyroid hormone binds to a thyroid hormone receptor bound to a thyroid hormone response element (TRE) on the target gene. This binding facilitates transcription of the gene and subsequently an increase in target protein expression (53).

Thyroid hormones regulate many processes involved in metabolism in the body. They increase the rat of glucose uptake into muscle cells via increased GLUT4 transport (54). Thyroid hormone has even been shown to ameliorate the hyperinsulinemia in Zucker Diabetic Fatty (ZDF) rats by inducing GLUT4 protein expression in red and white skeletal muscle (55). In the red skeletal muscle, the induction of GLUT4 is transcriptional, whereas in white skeletal muscle, it seems to be due to a posttranscriptional mechanism (55). A low affinity thyroid hormone receptor binding site was found and characterized within the GLUT4 promoter gene, providing a possible mechanism for direct stimulation of GLUT4 transcription by thyroid hormone (54).

Thyroid hormone also increases fatty acid oxidation and metabolism in skeletal muscle (57). In hepatocytes it has been observed that thyroid hormone increases the expression of PGC-1alpha and beta isoforms, and that both of these isoforms in turn

increase thyroid hormone's induction of CPT-1, acting as coactivators for thyroid hormone (58, 59). Thyroid hormone also plays an important role in mitochondrial biogenesis. Thyrotoxicosis induces increased expression of mitochondrial proteins, such as citrate synthase, cytochrome c, cytochrome oxidase, α-glycerophosphate dehydrogenase, and 3-hydroxybutyrate dehydrogenase, in skeletal muscle, especially the soleus and red quad (60, 61).

Thyroid Hormone and AMPK

Just from the brief overview given in this paper, it is clear that AMPK and thyroid hormone regulate some similar processes. Knowing these similarities, Winder and Hardie *et al* designed an experiment to see if AMPK was influenced by thyroid hormone (1). They found that all of the subunits of AMPK were increased in skeletal muscle, especially in the soleus and red quadriceps, with thyroid hormone treatment. There was also an increase in phospho-ACC, a marker of AMPK activity. At this point, however, the AMPKK complex had not yet been characterized, so the method behind the increase in AMPK activity remained unclear.

Proposal

AMPK and thyroid hormone share many similar metabolic effects, including fatty acid oxidation, glucose uptake, and mitochondrial biogenesis. The subunits of AMPK have also been shown to increase in response to thyroid hormone treatment, and they decrease in the absence of thyroid hormone, providing evidence for a possible regulatory role for thyroid hormone in AMPK-mediated pathways. There is also an

increase in AMPK activity associated with thyroid hormone treatment. We will examine whether or not the increase in AMPK activity is due to a thyroid hormone-induced increase in LKB1, MO25, and STRAD. We hypothesize that LKB1, MO25, and STRAD will increase in response to thyroid hormone treatment.

AMPK is activated by contraction, as has been said, and electrical stimulation of muscle can simulate contraction and activate AMPK (62). Since it is known that AMPK expression increases with thyroid hormone treatment, and since we hypothesize that LKB1-STRAD-MO25 complex proteins will increase, it is logical to further hypothesize that electrical stimulation of the muscle will cause an even greater increase in AMPK activity in hyperthyroid rats. More substrate (AMPK) and more kinase (AMPKK) under conditions of increased AMP/ATP ratio should result in more product formation.

Rat Treatment Protocol & Tissue Homogenization

All procedures are approved by the Institutional Animal Care and Use Committee of Brigham Young University. 30 male Sprague-Dawley rats (Sasco, Wilmington, MA) will be housed in a temperature controlled (21-22°C) room that cycles daily with a 12-h light and 12-h dark period. Rats will be kept in individual, hanging cages. Bedding will be replaced two times a week and all rats will be weighed every few days to keep a log of rat growth rate. Rats will be divided into three treatment groups. The first group (control) will receive powdered food and water ad libitum. The second group will receive 0.01% proplythiouracil in their drinking water to inhibit thyroid hormone synthesis. The third group will receive powdered Harlan Teklad rodent diet containing 3 mg of thyroxine and 1 mg of 3,5,3'-triiodothyronine per kilogram for 4

weeks. The night before we sacrifice the rats, they will receive the same amount of food, which will be determined by their feeding habits. They will be anesthetized by sodium pentobarbital injection (35-50 mg/kg ip). The soleus muscle, the gastrocnemius muscle, the red and white regions of the vastus lateralis muscle, and the heart will be quickly removed. Prior to removal of the left soleus and gastrocnemius muscle, the left tibial nerve will be electrically stimulated for five minutes at 1/sec to activated AMPK. Once removed, tissues will be compress frozen with metal tongs at liquid nitrogen temperature (appx. -196°C), wrapped immediately in labeled aluminum foil and then placed in a container filled with liquid nitrogen until they are stored later in an ultra-low temperature (-90°C) freezer. All tissues will be ground with a mortar and pestle at liquid nitrogen temperature and then homogenized in a homogenization buffer containing 250 mM mannitol, 50 mM Tris-HCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, and 1% triton all at a pH of 7.4. Just prior to homogenization 1 mM Dithiothreitol (DTT), 1 mM Benzamidine, 1µg/ml soybean trypsin inhibitor, and 0.5 mM AEBSF are added to the buffer. The final concentration of the tissue in the homogenization buffer solution will be a 9 factor dilution (1 g muscle powder / 9 ml buffer). Approximately 300 µl of raw homogenate will be taken from the homogenate and the rest will then be centrifuged at 1200 rpm for 20-m at 4°C. After the centrifugation, the supernatant from the homogenate will be immediately taken from the tubes placed into 1500 µl Eppendorf tubes.

Anticipated Experiments and Methods

Western Blots - Blots will be run on homogenized soleus, red quadriceps, white quadriceps, gastrocnemius, and heart of control, hyperthyroid, and hypothyroid rats to test for LKB1, STRAD, MO25, and PGC-1. In the stimulated soleus and gastrocnemius, we will blot for phospho-AMPK and phospho-ACC. These blots will enable us to see if AMPKK complex protein changes in response to thyroid hormone treatment. The phosphoproteins in the stimulated muscles will allow us to determine AMPK activation and activity variations among the groups.

Western Blot Protocol - Samples (raw homogenate / supernatant) are mixed with 1 X loading buffer that has been heated to 50°C for appx. 5-m. The samples are then loaded into wells of an appropriate percent Tris-HCl, ready-made Criterion® gel. The proteins in the sample are then separated via SDS-PAGE for 45-55 minutes at 200 V, depending on the percent gel. Immediately after electrophoresis, gels are rinsed in Transfer Bx (4°C) and properly placed in a protein transfer sandwich (fiber paper, filter paper, gel, PVDF membrane, filter paper, fiber paper). Proteins will then be transferred to PVDF membrane in mixing temperature controlled (4°C) Transfer Bx for 1-h at 100 V. Blocking solution (1.25 non-fat dried milk / 25 ml PBST) is prepared (pH 7.6) while transferring is taking place. Immediately following transfer, membranes are stained with PONCEAU to confirm a successful transfer, washed and then allowed to incubate in blocking solution for 1-h at rm. temperature. After blocking, the membrane is washed for 30-m with PBST and PBS and then the membrane is incubated overnight with the primary antibody at 4°C. The next day the membrane is washed for 30-m with PBST and PBS and then allowed to incubate for one more hour with the correct secondary

antibody with a Horseradish Peroxidase (HRP) tag. Immediately following incubation, the membrane is washed one last time for 30-m with PBST and PBS. The membrane is then exposed for 1 minute to a hydrolytic detecting solution (ECL) which will react with HRP and produce light on the membrane. Last of all, the membrane is quickly exposed to developing film in a dark room for the necessary time and the film is then developed, revealing the blot pattern. All blots will be summarized on blot reports and significance will be calculated.

Muscle Glycogen Assay- Glycogen is stored in both the liver and muscle tissue. The liver has the highest concentration of glycogen, and it can break down the glycogen into glucose, which can be passed into the blood stream for other tissues, especially the brain, to use. Muscle cells do not have the ability to pass glucose directly into the blood stream; they can break down glycogen for their own use, or they can convert it into lactate and pass that into the blood for hepatic gluconeogenesis. The levels of glycogen will indicate how much glycogen is stored in the treated rats. The likely decreased levels of glycogen stores in hyperthyroid rats will confirm that the metabolic rate is increased, and that more glycogen breakdown is needed for gluconeogenic substrates.

Muscle Glycogen Assay Protocol - Frozen tissue samples will be ground to powder under liquid nitrogen with a mortar and pestle. 100 mg of ground tissue will be weighed out and incubated for 30-m in 0.5 ml cold 30% KOH with a marble cap. After 30-m the muscle mixture will be neutralized with 10.7 M acetic acid (~.55 ml). Once neutralized, 0.1 ml of mixture will be transferred to a 12 X 75 mm tube containing 0.9 ml

amyloglucosidase buffer, capped and then incubated for 1-h at 55°C. Tubes are then centrifuged for 10-m prior to 0.1 ml being transferred to a cuvette containing 0.88 ml reaction buffer for an absorbance reading. Absorbance is read at an O.D. of 340 nm on a spectrophotometer. 15-m after the first readings, a final absorbance reading is measured with the addition of 0.02 ml G6PDH and HK enzyme mixture that has been mixed by inversion. From the O.D. results we will then be able to calculate μmoles glucose (from glycogen)/g tissue.

AMPK Activity Assay- The AMPK activity assay serves to test the hypothesis that the increase is AMPK activity in hyperthyroid rats is due to an increase in the expression and availability of LKB1, STRAD, and MO25. Since it is known that AMPK expression is increased in hyperthyroid rats, and since we hypothesize that AMPKK will increase, then there should be a greater increase in AMPK activity upon electrical stimulation. The AMPK activity assay will allow us to measure that activity, and see the difference between treatment groups.

AMPK Immunoprecipitation and Activity Assay Protocol - The two-step process of the standard AMPK activity assay includes the immunoprecipitation of AMPK and then the subsequent phosphorylation of SAMS peptide by phospho-AMPK. AMPK is immunoprecipitated by incubating anti α2 AMPK antibody with Protein G-sepharose for one hour. It is then resuspended in immunoprecipitation buffer (IP buffer). When the sepharose/antibody mix is adequately resuspended and homogeneous, then 10 μl are aliquotted into 1.5 ml Eppendorf tubes along with 50 μl of tissue homogenate. This

mixture is incubated overnight to allow immunoprecipitation of AMPK. The next day, the mixture is washed with IP buffer and salt to remove all nonspecific proteins. It is then resuspended with 30 µl Hepes buffer. 10 µl are then pipetted into 12X75 test tubes. We will be able to measure activity of the precipitated AMPK substrate through the incorporation of a radioactive phosphate group (^{32}P) from ATP into the SAMS peptide serving as the AMPK substrate. 25 µl of phosphorylation buffer will be added to each test tube, and the reaction will end by spotting 1-cm^2 pieces of pre-cut P81 filter paper (Whatman, Tewksbury, MA) with 15 ul of the final reaction mixture after 10-m. Finally, the filter papers will be washed 6 times in 250 ml 1% phosphoric acid. The phosphoric acid will be neutralized with Sodium Bicarbonate while the Whatman papers are rinsed with acetone and allowed to dry. After the papers are dry they will be placed in 3 ml of Ecolite (ICN, Irvine, CA) and then counted for 5 minutes on a scintillation counter.

Buffers

Often DTT, Benzamidine, AEBSF and soybean trypsin inhibitor will be added to buffers just prior to use in order to inhibit protease activity.

Muscle Homogenization Buffer: 250 mM mannitol, 50 mM Tris-HCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1% triton pH adjusted to 7.4.

4 x Loading Buffer (92% stock soln.): 240 mM Tris-HCl, 8% SDS (sodium dodecyl sulfate), 40% Glycerol, .04% bromophenol blue all at a pH of 6.8. Add 8% β-mercaptoethanol just prior to use to bring the buffer to 100% stock solution.

1 x Transfer Buffer (20% MeOH): 48 mM TRIS, 39 mM Glycine, .074% SDS, 20% Methanol, pH 9.0.

100 mM Tris Buffer, pH 8.0: 4.02 grams Trizma HCl + 2.97 grams Trizma Base diluted to 500 ml with distilled water. Adjust to final pH with HCl or KOH.

***Amyloglucosidase buffer*:** (fresh daily - keep cold): Add 100 μg amyloglucosidase to 1 ml distilled water. Allow to dissolve. Add 0.1 ml from this solution to 10 ml, 50 mM acetate buffer pH, 4.7.

***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, 0.05 μCi/μl [γ^{32}]ATP, pH 7.0.

1 X Phosphate-buffered Saline (PBS): 138.6 mM NaCl, 2.71 mM KCl, 2.15 mM KH_2PO_4, 9.89 mM Na_2HPO_4, pH 7.3.

1 X PBST: Same as PBS but add 0.1% Tween20.

IP Buffer: 50 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 5mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 ug/ml soybean trypsin inhibitor, pH 7.4.

Hepes Buffer: 25 MM HEPES, 0.02% Brij, 1 mM DTT, pH 7.0.

Stock Solution: 100 mM HEPES, 200 mM NaCl, 20% glycerol, 2 mM EDTA, 12.5 mM $MgCl_2$, pH 7.0.

Working Assay Cocktail: 0.5 mM AMP, 2 mM DTT, 0.5 mM ATP in stock solution. 1 mM SAMS peptide and 10 mCi/ml P^{32}-ATP is added just before running the assay.

Estimated Costs

The cost of analysis for the experiments should amount to approximately the following values:

Western Blots-

- *Developing reagents* ~ $4,000.
- *Antibodies net worth* ~ $4,500.

Muscle Glycogen Assay ~ $100.

AMPK Activity Assay ~ $2000

- P^{32}*-ATP* ~ $300

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Abstract

AMP-activated protein kinase (AMPK), a heterotrimeric protein which serves as a metabolic master switch in skeletal muscle, is a research target for the pharmaceutical treatment and prevention of type 2 diabetes. The expression of all of the isoforms of the subunits of AMPK and AMPK activity is increased in skeletal muscle tissue of hyperthyroid rats. Activity of AMPK is regulated by an upstream kinase (AMPKK). The LKB1-STRAD-MO25 complex is a major AMPKK in skeletal muscle. This experiment was designed to determine whether the increase in AMPK activity is accompanied by a thyroid hormone-induced increase in the expression of the LKB1-STRAD-MO25 complex. LKB1-STRAD-MO25 complex protein expression was determined by Western blots in control rats, in rats given 3 mg of thyroxine and 1 mg of triiodothyronine per kilogram chow for 4 weeks, and in rats given 0.01% propylthiouracil (PTU) in drinking water for 4 weeks. The relative expression of LKB1, MO25, and STRAD, as well as PGC-1α, increased in the soleus of thyroid hormone treated rats vs. the controls. MO25 mRNA increased with thyroid hormone treatment, and STRAD mRNA increased with PTU treatment. Phospho-AMPK and phospho-ACC increased in response to electrical stimulation in muscles of all treatment groups, but was most markedly increased in hyperthyroid rats. Thyroid hormone treatment also increased the amount of phospho-CREB in the soleus, heart, and red quadriceps. These data provide evidence that thyroid hormone partially controls expression of the LKB1-STRAD-MO25 complex, as well the subsequent activation of AMPK.

Key Words: AMPK, LKB1, CREB, Acetyl-CoA carboyxlase, PGC-1α

THESIS

Introduction

5'-AMP-activated protein kinase (AMPK) acts as a fuel gauge for the cell, responding to changes in cellular energy (10, 41). When it "senses" low ATP, it turns off ATP-depleting pathways and switches on mechanisms of ATP production (10, 41). AMPK is a heterotrimer consisting of a 63-kD alpha subunit, a beta subunit, and a gamma subunit (11). There are at least two isoforms each of the alpha and beta subunits, and three isoforms of the gamma subunit (10, 41). AMPK is activated during exercise, ischemia, and hypoxia, and other forms of cellular stress (31). AMPK senses increases in the AMP/ATP ratio and the creatine/creatine phosphate (CP) ratio (31). AMPK is allosterically activated by AMP, and inhibited by ATP (10, 39), which allows for its sensitivity to cellular energy. ATP inhibits AMPK by binding to the Bateman domains on the gamma subunit (10). AMP is reported to activate AMPK via three distinct pathways. The first pathway involves allosteric activation of AMP via binding to the gamma subunit (10, 13). AMP, when bound to the gamma subunit, also makes AMPK a poor substrate for protein phosphatase 2C (30, 39). The third pathway, that AMP-binding makes AMPK a better substrate for AMPK kinase (AMPKK) is currently in dispute (14, 30, 34, 45).

LKB1, STE20-related adaptor protein (STRAD), and mouse protein 25 (MO25) form a complex which makes up the upstream kinase for AMPK, with LKB1 in possession of the catalytic domain (2, 3, 12). LKB1 is a serine/threonine kinase, which has been shown to be a tumor suppressor (2, 3, 12, 38). Mutations in LKB1 are linked to Peutz-Jeghers syndrome, in which multiple gastrointestinal polyps form and the

likelihood of malignant tumors is greatly increased (2, 16). LKB1 alone is mainly localized in the nucleus, but when associated with STRAD and MO25, it is anchored in the cytoplasm (2, 3). STRAD and MO25 have no catalytic function (3). Association between LKB1 and STRAD is enhanced by increasing levels of MO25 in a dose-dependent fashion. Optimal activation of AMPK by LKB1 requires the presence of all three proteins (2, 3, 12).

Aware of the similarities between the effects of thyroid hormone and those of AMPK, Park *et al*. (27) designed an experiment to see if AMPK was influenced by thyroid hormone. They found that all of the subunits of AMPK were increased in skeletal muscle, especially in the soleus and red quadriceps, with thyroid hormone treatment. There was also an increase in both acetyl-CoA carboxylase (ACC) and phospho-ACC. Malonyl-CoA concentration was reduced in thyroid hormone treated rats. At this point, however, the AMPKK complex had not yet been characterized, so the mechanism of the increase in AMPK activity remained unclear. In an effort to see the effect of thyroid hormone upon these AMPKK complex proteins, which are now characterized, we designed an experiment similar to that of Park *et al*. (27), hypothesizing that, just as the AMPK protein subunit expression increased with thyroid hormone treatment, so too, would the expression of LKB1, STRAD, and MO25.

Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) is a transcriptional regulator for genes involved in mitochondrial biogenesis, fatty acid oxidation, and gluconeogenesis (28). PGC-1α enhances the activity of transcription factors like nuclear respiratory factor 1 (NRF-1), myocyte enhancer factor 2 (MEF2), host cell factor (HCF), and others (1, 24). It also has a positive feedback loop, enhancing

its own expression (8). Both a MEF2 site and cAMP response element (CRE) are essential for contraction-induced PGC-1α promoter activity (1). AMPK is required for increased PGC-1α expression in skeletal muscle in response to creatine depletion (47). LKB1 knockout mice show a decrease in PGC-1α, as well as mitochondrial proteins (37). Irrcher *et al. (19)* found that PGC-1α expression increased in skeletal muscle after 5 days of thyroid hormone treatment, the largest increase occurring in the soleus.

Cyclic AMP response element binding protein (CREB) is a well-characterized transcription factor activated by various stimuli (33). It was originally discovered as a downstream target of the cAMP pathway. CREB is activated by phosphorylation at the serine 133 position (6, 9). CREB is involved in a variety of cellular processes such as proliferation, development, and differentiation. It is also involved in metabolic processes, such as regulation of gluconeogenesis by glucagon and epinephrine (9, 33). Mendez-Pertuz *et al.* (23) found that treatment with thyroid hormone will decrease the phosphorylation of CREB by PKA at the serine 133 position in pituitary cells. On the other hand, Ghosh *et al.* (5) reported that thyroid hormone influences the differentiation and maturation of astrocytes via activation of the PKA pathway. Due to recent experiments in our lab (W. Winder, unpublished data), showing that AMPK and PKA both phosphorylate CREB at the serine 133 position, and knowing that PGC-1α, whose gene sequence contains a CRE, increases in thyroid hormone-treated rats, we hypothesized that CREB phosphorylation would increase in skeletal muscle upon treatment with thyroid hormone (19, 22).

Materials and Methods:

Animal care. All procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Male Sprague-Dawley rats (Sasco, Wilmington, MA) were housed in individual cages in a room lighted from 6 AM to 6 PM. Rats were fed powdered Harlan Teklad rodent diet (Madison, WI). Rats weighed an average of 107 ± 20.6 g at the beginning of treatments. Rats were divided into three treatment groups. The first group (controls) received powdered food and water ad libitum. The second group received 0.01% propylthiouracil (PTU) in drinking water for 4 wk for the purpose of inhibiting thyroid hormone synthesis. The third group was provided with powdered Harlan Teklad rodent diet containing 3 mg of thyroxine and 1 mg of 3,5,3'-triiodothyronine per kilogram for 4 wk. On the afternoon before the rats were killed, they were given 20 g of rat chow.

Muscle stimulation, collection, and homogenization. Rats were anesthetized with pentobarbital sodium (0.08 mg/g body wt) for at least 30 min before beginning the procedure. The right soleus and gastrocnemius were isolated and frozen using aluminum block tongs at liquid nitrogen temperature. The left tibial nerve was isolated and stimulated at a frequency of 1/s, 10-ms duration, 15 volts, for 5 min. The left gastrocnemius and soleus were then removed and clamp-frozen. The red and white quadriceps, heart, and liver were also removed at this time. Tissues were kept frozen at −95°C until analyses. The retroperitoneal fat pads were weighed as an indicator of adiposity. Muscles were weighed and then homogenized in 9 vol of homogenization

buffer (50 mM Tris·HCl, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, and 5 µg/ml soybean trypsin inhibitor; pH 7.4). The homogenate was centrifuged at 1,200 *g* and 4°C, and the supernatant was kept for analysis.

Western blotting and immunodetection. Homogenates were diluted in sample loading buffer (50 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.1% bromphenol blue) and loaded (4 µl homogenate) on 5% [phospho (p)-ACC,PGC-1], 7.5% (p-AMPK, PGC-1, LKB1), or 10% (pCREB, CREB, MO25, STRAD) Tris·HCl gels (Bio-Rad Criterion System, Hercules, CA.). Electrophoresis was applied for 53 min at 200 volts, after which the proteins were transferred to polyvinylidene difluoride membranes at 100 volts for 1 h. Membranes were stained with Ponceau S to ensure even transfer and protein loading across lanes then washed in Tris-buffered saline plus Tween (TBST), blocked for 1 h at room temperature in 5% nonfat dry milk in TBST, and probed overnight at 4°C with primary antibody diluted in 1% BSA dissolved in TBST. Commercially available antibodies and dilutions were as follows: LKB1, p-ACC and p-AMPK (1:1000, 1:750 and 1:1000 resp.; Cell Signaling Technology, Beverly, MA); STRAD and MO25 (1:20,000; Affinity Bioreagents, Inc, Golden, CO); CREB and PGC-1 (1:5000 and 1:1,000; Chemicon International, Temecula, CA); and p-CREB (1:2000; Upstate, Lake Placid, NY). After primary antibody incubation, the membranes were washed in TBST and incubated for 1 h at room temperature in HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in 1% nonfat dry milk in TBST. Membranes were again washed in TBST and then covered

with enhanced chemiluminescence Western blotting detection reagent (GE Healthcare Biosciences Corp) for 1 min. HRP activity was then detected using autoradiographic film (Classic Blue Sensitive; Midwest Scientific, St. Louis, MO). Relative band intensity was quantified using the Spot Denso function of AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

Real-time PCR. Relative changes in mRNA with training were examined by real-time PCR. Total mRNA was isolated from 20-30 mg of tissue from control, thyroid hormone treated rats, and PTU treated rats with the RNeasy fibrous tissue kit (Qiagen) according to the manufacturer's instructions. Samples were homogenized with an Ultra-Turrax T8 (IKA, Wilmington, NC). cDNA libraries for each sample were generated using the SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR using gene-specific primers for LKB1, MO25, and STRAD mRNA and 18S rRNA was performed, using the Platinum SYBR Green qPCR Supermix UDG kit (Invitrogen) with an ABI-7000 real-time PCR System. Primers (Invitrogen) were the following: LKB1 forward, AGAGGAAGTGGGTCAGAATGGA; LKB1 reverse, CCGGCCTTCTGGCTTCA; MO25 forward, GGTTGCCATGAAAGAAATTCTGT; MO25 reverse, AGCTGTGCTACGGCCTCTGT; STRAD forward, TCCGAGGCTTTACCTGAGTTG; STRAD reverse, AGACTGGCTGCCCTCGAA; 18S forward, GTGCATGGCCGTTCTTAGTTG; and 18S reverse, GCCACTTGTCCCTCTAAGAAGTTG. Samples were amplified in triplicate. Amplification protocol was 50°C for 2 min and 95°C for 10 min and then 60 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s. For postamplification dissociation to

generate melting curves, temperature was raised from 60°C to 95°C. Cycle threshold (C_T) was calculated automatically by the ABI software. Relative fold expression was calculated using the $2^{-\Delta\Delta CT}$ method after normalizing to 18S RNA (21).

Results

Evidence of hyper- and hypothyroid conditions.

The final body weights for the control, thyroid, and PTU were 283 ± 11, 249 ± 12, and 228 ± 8 g respectively. Final body weights for both thyroid and PTU rats were significantly less than the control ($P < 0.05$). As evidence that the PTU was indeed inhibiting thyroid hormone production, there was marked hypertrophy of the thyroid in PTU treated rats (71.0 ± 8.6 mg vs. 19.0 ± 2.2 mg in controls). The retroperitoneal fat pad weight expressed as a percentage of body weight for the control, thyroid, and PTU were 0.58 ± 0.04, 0.08 ± 0.01, and 0.39 ± 0.03, respectively. The fatpad weight for thyroid and PTU rats were both significantly less than the control, and the thyroid rats were significantly less than both controls and PTU rats ($P < 0.05$). The heart weights for the control, thyroid, and PTU rats were 0.79 ± 0.02, 1.36 ± 0.07, 0.58 ± 0.02 g respectively. The thyroid and PTU heart weights were significantly different from controls ($P < 0.05$). Expressed as a percentage of body weight, the heart weights for the three groups were, respectively, 0.28 ± 0.01, 0.55 ± 0.02, and 0.25 ± 0.01 with the thyroid group being significantly different from both ($P < 0.05$). During the last week before the animals were sacrificed, the average daily food intake for control, thyroid, and PTU was 30.7 ± 0.4, 40.0 ± 1.3, and 22.9 ± 1.1 g respectively. Both thyroid and PTU groups were significantly different from controls.

LKB1, STRAD, and MO25 Western Blot in skeletal muscle

LKB1, STRAD, and MO25 migrated in the gel to the approximate positions based on their molecular weight (LKB1 at 55 kD, STRAD at 48 kD, and MO25 at 40 kD). Figure 1A-C shows that all LKB1, STRAD, and MO25 were present in significantly higher amounts (150%, 200%, and 160% increases respectively compared to the control) in the thyroid rats than in control rats in soleus muscle ($P < 0.05$). In heart, red quad, white quad, and gastrocnemius muscle, MO25 expression increased approximately 140%, 150%, 125%, and 210% respectively in the thyroid hormone treated rats ($P < 0.05$). LKB1 and STRAD expression varied among the different muscle types (see Table 1).

Phospho-ACC and phospho-AMPK Western Blot in resting and stimulated skeletal muscle.

A previous study showed that both ACC and phospho-ACC increase in skeletal muscle due to thyroid hormone treatment (12). To test whether thyroid hormone treatment would increase the amount of AMPK phosphorylation as well as activation, as shown by the subsequent phosphorylation of ACC, we stimulated the left tibial nerve for 5 minutes. Both gastrocnemius and soleus muscles showed an increase in phospho-AMPK under resting conditions (see Fig 2). Figure 3 shows that, upon stimulation, the relative phosphorylation of ACC increased approximately 60% more in the gastrocnemius of thyroid hormone treated rats (increase compared to the average resting control rat phospho-ACC levels), than in the control rats. The relative amount of

phospho-AMPK did not increase significantly in the gastrocnemius of thyroid hormone treated rats, but there was a trend to increase (data not shown). Also, when the amount of phospho-AMPK was compared only in the stimulated gastrocnemius, there was 70% more phospho-AMPK in thyroid hormone treated rats than in the PTU rats and 45% more phospho-AMPK than in the control rats, although the difference from the control rat tissue was not significant. The phospho-ACC level in the stimulated gastrocnemius of thyroid hormone treated rats was about 60% higher than in the stimulated gastrocnemius of control rats.

CREB and PGC-1α Western Blot in skeletal muscle

CREB protein expression decreased by 20% in heart muscle in PTU rats compared to the other groups. CREB did not change among the treatment groups for the other muscle types (see Table 2). Figure 4 shows that PGC-1α expression was 40% higher in the soleus of thyroid hormone treated rats than that of the control rats ($P < 0.05$). There was no significant change in any of the other muscle types (data not shown).

Phospho-CREB Western Blot in resting and stimulated muscle

Under resting conditions, phospho-CREB levels were significantly higher in thyroid hormone treated rats in the soleus, heart, and red quadriceps (120%, 70%, and 40% higher than control respectively) than in the control rats (see Fig 5). There was a trend to increase in the gastrocnemius, but the increase was not significant. Upon electrical stimulation, however, there is a two-fold increase in CREB phosphorylation in

the gastrocnemius of the thyroid hormone treated rats. In control and PTU treated rats, there is no significant increase in phospho-CREB upon stimulation (see Fig 5D).

LKB1, STRAD, and MO25 mRNA expression

Relative levels of LKB1, STRAD, and MO25 mRNA were measured in the soleus of control, PTU, and thyroid hormone treated rats. LKB1 mRNA levels did not change with thyroid hormone or PTU treatment. MO25 mRNA increased approximately to 170% of the control with thyroid hormone treatment. STRAD mRNA did not increase with thyroid hormone treatment, but, surprisingly, it increased to about 150% of the control with PTU treatment (see Fig 6).

Discussion

A previous study from this lab (27) showed that AMPK is regulated, at least in part, by thyroid hormone. All isoforms of the subunits of AMPK were influenced by thyroid hormone and PTU treatment. There was also a decrease in malonyl-CoA content in muscles of thyroid hormone treated rats. ACC and phospho-ACC increased in response to thyroid hormone treatment, and the phosphorylation of ACC corresponded to the amount of ACC. Due to the changes in malonyl-CoA and ACC activity, it seemed that thyroid hormone could regulate the activity, as well as the amount, of AMPK.

The AMPKK complex proteins were not characterized until 2003 (3, 12), and it was therefore impossible at the time of Park's (27) study to determine the effect of thyroid hormone on LKB1, STRAD, and MO25. It seemed likely that the expression of these proteins would increase in response to thyroid hormone treatment just as the

subunits of AMPK had. This proved to be the case, although the increase in LKB1, STRAD, and MO25 seemed more tissue dependent than the changes in AMPK subunit expression. LKB1, STRAD, and MO25 all increased significantly in the soleus of thyroid hormone treated rats. MO25 increased significantly in all other tissue types analyzed – heart, gastrocnemius, red quadriceps, and white quadriceps.

The mechanism by which LKB1, STRAD, and MO25 expression increased in this study is unknown. Since thyroid hormone generally elicits a cellular response by means of binding to a receptor in the nucleus, it would seem likely that thyroid hormone would increase transcription of the genes which encode for LKB1, STRAD, and MO25. It seems that this is not necessarily the case. MO25 mRNA increased to 170% of control levels in response to thyroid hormone treatment, following the trend observed by Western Blot, but LKB1 and STRAD did not increase significantly. STRAD mRNA did increase to 150% of control levels, however, in the soleus of the PTU treated rat.

In order to further delineate the mechanism by which thyroid hormone regulates the increase in MO25 mRNA, and by which the lack of thyroid hormone increases STRAD mRNA, we looked for possible thyroid hormone response elements in promoter regions for the genes encoding for these proteins. We found a possible, although weak, thyroid hormone response element (TRE) in human chromosome 19 on the possible promoter region for the STK11 gene proposed by Hearle *et al. (7, 15)*. It consists of a single AGGTCA sequence, so its ability to function is questionable (NCBI Accession # AC011544). We also saw a potential TRE (GGATCACCTGAGGTCA) about 1500 kb upstream of the start sequence for STRAD on the LYK5 gene (NCBI Accession # AC015651). We did not find a TRE sequence upstream of the gene encoding for MO25

(NCBI Accession # AC084031). It is possible, therefore, that thyroid hormone binds to the TRE's we located to regulate the increase in expression of LKB1, STRAD, and MO25, but, since no definitely functional TRE was found for these genes, it is more probable that thyroid hormone regulates the transcription of genes which encode for other transcriptional regulators, coactivators, or corepressors.

It is interesting that MO25 mRNA was the only one of the three analyzed that increased, although the protein expression for all three increased in the soleus of thyroid hormone treated rats. We observed similar results in our lab in a study on endurance training, when we analyzed the red quadriceps of trained rat (36). LKB1 and MO25 protein expression increased, but only MO25 mRNA expression increased. So although the increase in MO25 mRNA observed in our current study may in part be responsible for the increase in MO25 protein expression, the mRNA for LKB1 and STRAD do not seem to be responsible for the increase in protein expression. It was suggested in our previous study (36) that LKB1 may increase due to protein-protein interactions, such as binding to HSP90 (4, 26), since HSP90 has been shown to increase LKB1 protein expression. It is unknown why STRAD mRNA increases in PTU treated rats, while the protein expression tends to decrease. It is possible that STRAD mRNA is quickly destabilized, so that only a smaller portion of it is translated into protein.

The left tibial nerve was stimulated for five minutes prior to removal in order to activate AMPK in the gastrocnemius (18). Since we hypothesized that LKB1, STRAD, and MO25 would increase in response to thyroid hormone treatment, and knowing from Park's study (27) that AMPK expression increases in response to thyroid hormone, it followed that upon electrical stimulation there would be a greater activation of AMPK

and subsequent phosphorylation of ACC. There was a greater increase in ACC phosphorylation upon stimulation in the thyroid hormone treated rat gastrocnemius, indicating that there was more AMPK activity. There was a higher content of phosphorylated AMPK in the stimulated muscle of thyroid hormone treated rats, although the relative phosphorylation (amount of phospho-AMPK in stimulated gastrocnemius compared to the resting control) did not significantly increase. It appears that thyroid hormone treatment results in increased phosphorylation and activation of AMPK, and when the muscle is electrically stimulated, there is a great amount of AMPK activation in thyroid hormone treated rats. The total amount of phosphorylated AMPK increases simply because there is more enzyme (LKB1, STRAD, MO25) and more substrate (AMPK). It seems logical, therefore, under conditions where AMPK can be activated, such as electrical stimulation, that if there is more AMPKK present and more AMPK, then there will be an increased amount of phospho-AMPK.

It is also interesting that an increase in phospho-AMPK was observed in the gastrocnemius, even though MO25 was the only AMPKK complex protein whose expression increased. It seems that MO25 is the most highly influenced by thyroid hormone treatment out of the three AMPKK complex proteins (there was a significant increase in expression in all tissue types analyzed). It is possible, therefore, that MO25 is responsible, at least in part, for the increase in AMPK activity observed upon treatment with thyroid hormone. This is further supported by the fact that MO25 was the only AMPKK complex protein showing increased expression increased in the gastrocnemius of thyroid hormone treated rats, yet an increase in AMPK activity was still observed in the gastrocnemius.

CREB is a transcription factor involved in numerous cellular and metabolic processes. It binds to cyclic AMP response element (CRE), where it can be activated by phosphorylation at the serine 133 position (20). This phosphorylation allows for recruitment of CREB binding protein (CBP) or p300, two similar proteins that have histone acetylase activity (HAT), as well as the ability to interact with basal transcription factors such as TFIIB and TATA-binding protein (TFB) (20, 32). There are CRE's on many different genes involved in cell proliferation, differentiation, and division, as well as metabolic regulations, and when CREB is activated, it can facilitate the transcription of these genes (25, 32, 33, 35). We observed an increase in phospho-CREB in the soleus, heart, and red quadriceps of thyroid hormone treated rats. Since the amount of CREB protein did not change due to thyroid state, except for a decrease in expression in the heart of PTU treated rats, it is evident that thyroid hormone treatment increases the amount of phosphorylation of CREB, and possibly its activation. This could occur through various different pathways, since CREB can be phosphorylated by a variety of proteins. The best known pathway is through PKA, but it is dependent on cAMP concentration, and cAMP levels have been shown to decrease in the soleus during thyrotoxicosis, it seems unlikely that this increase is PKA mediated (40). Another interesting observation was that phospho-CREB increases in the gastrocnemius upon electrical stimulation only in the thyroid hormone treated rats (see Fig 6D). It is possible that thyroid hormone prepares the muscle cell to quickly activate CREB upon muscle contraction.

It was not surprising to observe an increase in PGC-1α in soleus muscle of thyroid hormone treated rats. It simply confirmed what Irrcher *et al.* (19) found in their

study. It is also known that CREB induces expression of PGC-1α, at least in the liver (17). Since we observed that thyroid hormone treatment increased AMPK activity, as well as CREB phosphorylation, the increase in PGC-1α followed the trend. It was interesting to note, however, that although phospho-AMPK and phospho-CREB increased in various tissue types upon treatment with thyroid hormone, PGC-1α only increased in the soleus. The soleus muscle seems to be the muscle tissue type most heavily influenced by thyroid hormone, so it is unsurprising that an increase in protein expression would be seen there and not elsewhere (40, 43).

It is possible that the increase in LKB1, and subsequent activation of AMPK, by thyroid hormone could be partially responsible for some of thyroid hormone's effects on metabolism. Hyperthyroidism results in marked increases in glucose uptake, fatty acid oxidation, and oxidative capacity. The induction of PGC-1α has been shown in hepatocytes to increase, among other things, the induction of carnitine palmitoyltransferase-1 (CPT-1) which aids in the transport of fatty acids into the mitochondrion for oxidation (29, 46). AMPK has been shown to relieve the inhibition of CPT-1 by malonyl-CoA by inactivating ACC (42), so it seems possible that the increase in fatty acid oxidation observed in hyperthyroid patients occurs by increasing expression of LKB1, STRAD, and MO25, which leads to subsequent AMPK activation, as well as by increasing expression of PGC-1α.

Another effect of hyperthyroidism is increased oxidative capacity. It has been shown that thyrotoxicosis induces increased expression of mitochondrial proteins, such as citrate synthase, cytochrome c, cytochrome oxidase, α-glycerophosphate dehydrogenase, and 3-hydroxybutyrate dehydrogenase, in skeletal muscle, especially the

soleus and red quad (40, 43). AICAR stimulation of AMPK increases cytochrome c and δ-aminolevulinate synthase (ALAS), a rate-limiting enzyme involved in the production of heme. Malate dehydrogenase and succinate dehydrogenase also increase, as well as citrate synthase activity, in rats treated with AICAR injections (44). Conversely, in LKB1 knockout mice, there are decreases in cytochrome c and citrate synthase activity, even if the mice are "trained" by voluntary exercise (37). It is therefore conceivable that thyroid hormone-induced increases in LKB1-STRAD-MO25 complex proteins, and the ensuing activation of AMPK, are partially responsible for the thyrotoxic increase in oxidative capacity.

In conclusion, we found that thyroid hormone treatment does increase the protein expression of LKB1, STRAD, and MO25, as well as the activity of AMPK upon electrical stimulation. Thyroid hormone treatment also increases the phosphorylation of CREB in the soleus. These results suggest that thyroid plays at least some role in the regulation of AMPK activity and the activation of CREB in skeletal muscle.

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Figure Legends

Figure 1

Relative expression of A) LKB1, B) STRAD, and C) MO25 in control (cntrl), thyroid hormone-treated (hyper) and PTU-treated rats (hypo). LKB1 expression in hyper- and hypothyroid rats was significantly different from the control. STRAD and MO25 expression in hyperthyroid rats was significantly higher than in the controls ($n=9$, $p < 0.05$, * = significantly different from the control).

Figure 2

Activation of AMPK was measured by western blot showing relative quantities of phosphothreonine-172 AMPK (pAMPK) in A) resting gastrocnemius and B) resting soleus. There was significantly more phospho-AMPK in the hyperthyroid rats than the control for both gastrocnemius and soleus. There was significantly less phospho-AMPK in the soleus of the hypothyroid rats than the control, but there was no significant difference in the gastrocnemius ($n=9$, $p < 0.05$, * = significantly different from the control).

Figure 3

Activity of AMPK was determined by measuring the phosphorylation of ACC in stimulated muscle by western blot. 3A shows the amount of phospho-ACC in the stimulated muscle relative to the amount of phospho-ACC in the resting control. Phospho-ACC increases significantly in the stimulated gastrocnemius of all three treatment groups relative to the control, but the hyperthyroid rats have the most

markedly increased amount of phospho-ACC. 3B shows the relative amount of phospho-AMPK in the stimulated gastrocnemius (n=9, $p < 0.05$, * = significantly different from the control).

Figure 4

Relative expression of PGC-1α in control, hyper-, and hypothyroid rats was determined by western blot. Expression of PGC-1α in the soleus of hyperthyroid rats was significantly higher than the control (n=9, $p < 0.05$, * = significantly different from the control).

Figure 5

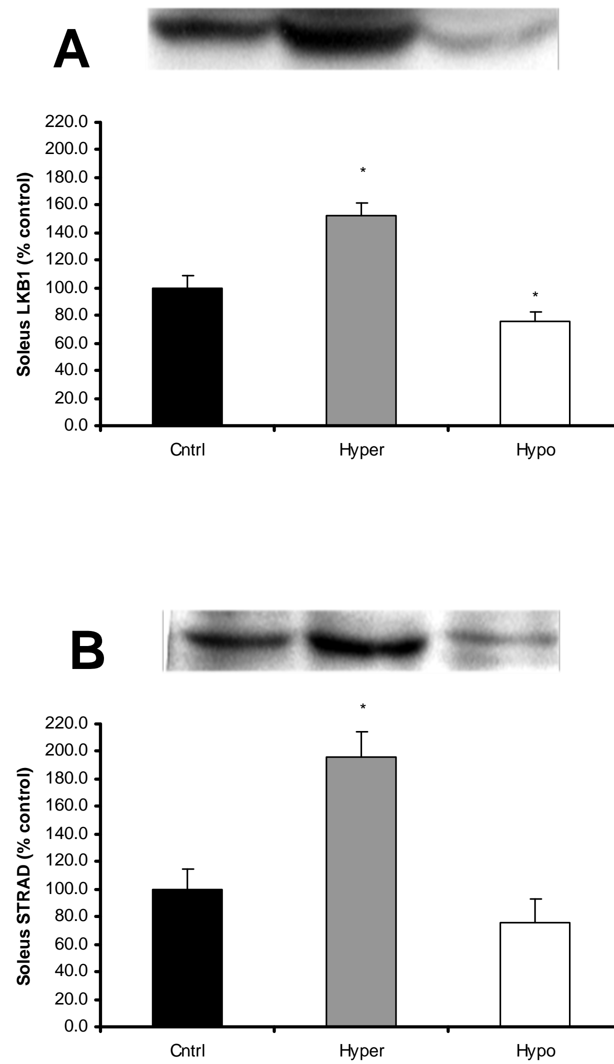
Activation of CREB was determined by western blot in control, hyper-, and hypothyroid rats showing relative expression of phosphoserine-133 CREB (pCREB) in resting A) soleus, B) heart, and C) red quadriceps. Phospho-CREB increased significantly in all three muscle types vs. the control. Phospho-CREB decreased significantly in the soleus and heart of hyperthyroid rats, but not in the red quadriceps. 5D shows the amount of phospo-CREB in the stimulated muscle relative to the amount of phospho-CREB in the resting control. Phospho-CREB increases significantly in the stimulated gastrocnemius only in the hyperthyroid rats (n=9, $p < 0.05$, * = significantly different from the control).

Figure 6

Relative amounts of mRNA in the soleus of control, hyper-, and hypothyroid rats for A) LKB1, B) STRAD, and C) MO25 were determined by real-time PCR. MO25 mRNA

increased significantly in hyperthyroid rats, whereas STRAD increased significantly in hypothyroid rats. There was no significant change in LKB1 mRNA (n=8, $p < 0.05$, * = significantly different from the control).

Figure 1.



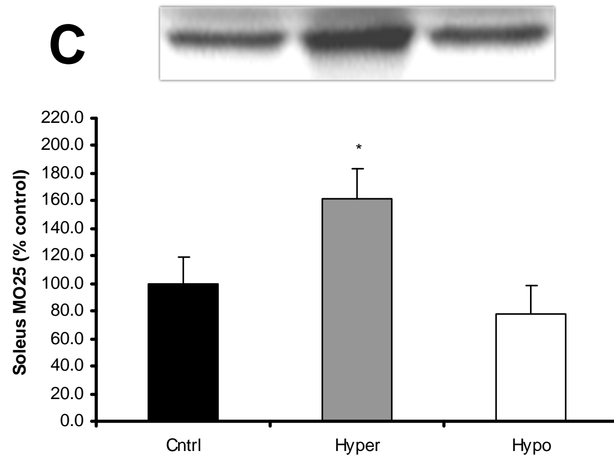
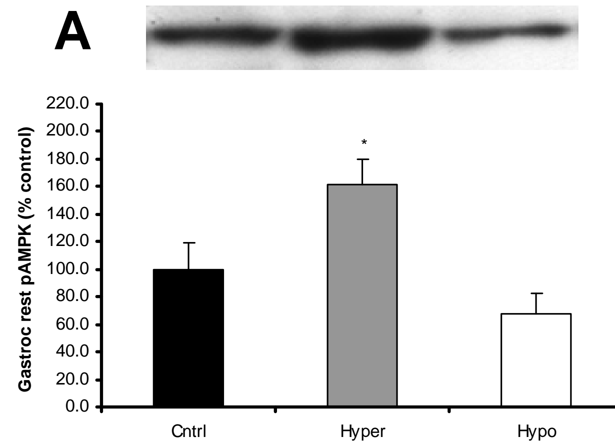

C
Soleus MO25 (% control)
220.0
200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
*
Cntrl
Hyper
Hypo

Figure 2.



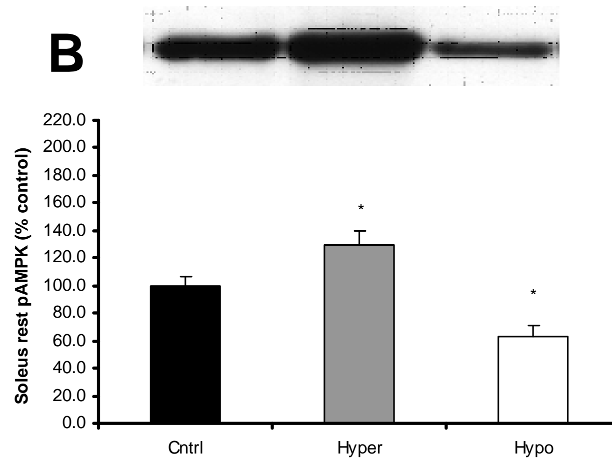


Figure 3.

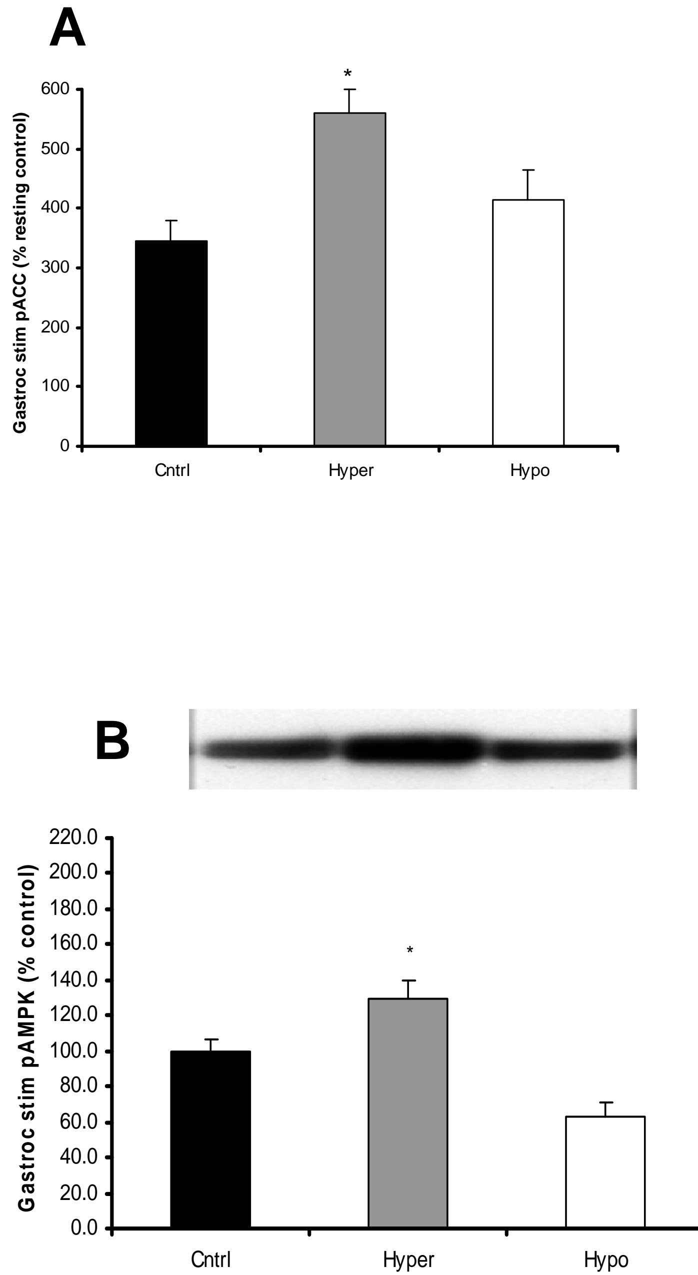


Figure 4.

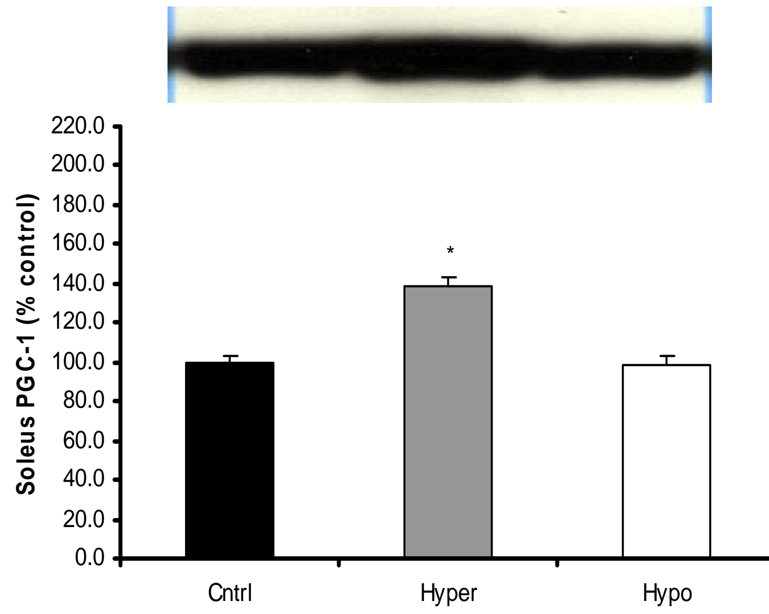
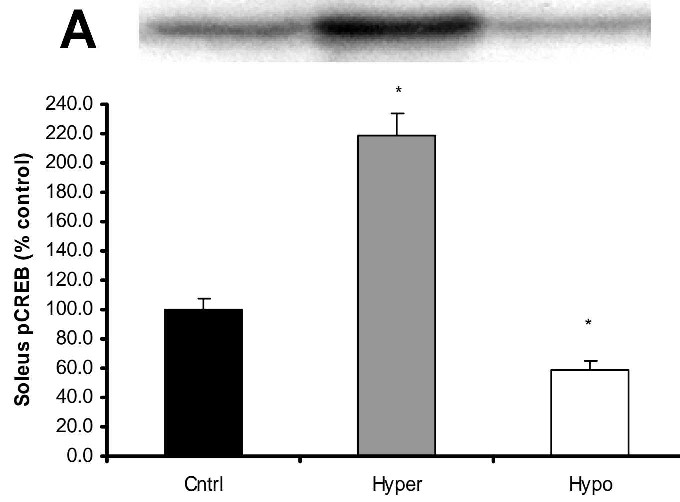
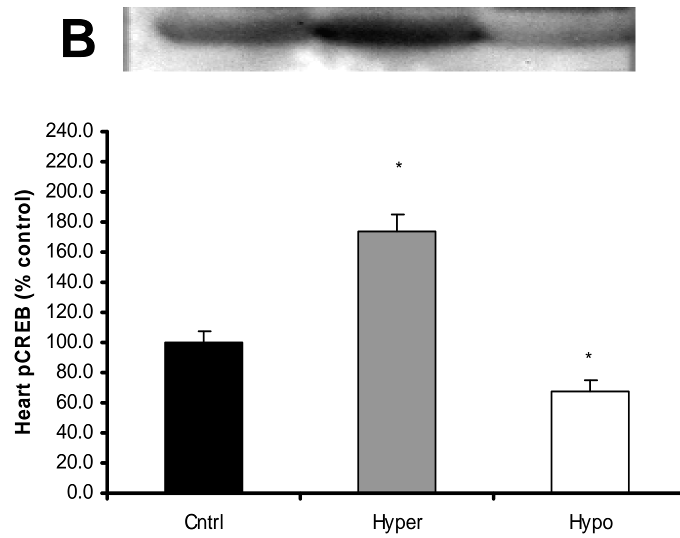
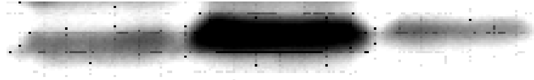


Figure 5.



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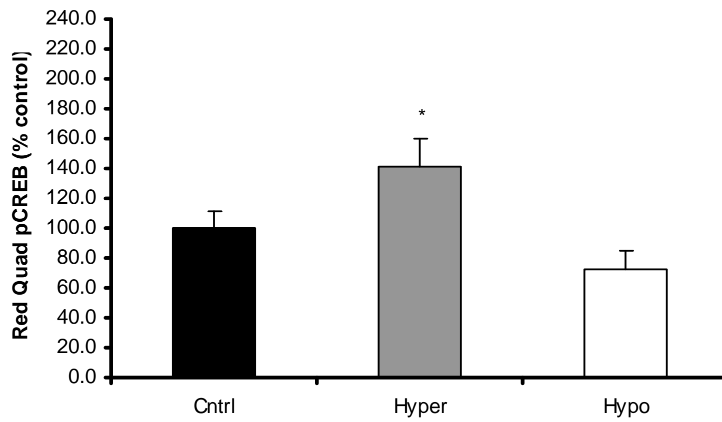

Red Quad pCREB (% control)
240.0
220.0
200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
*
Cntrl
Hyper
Hypo

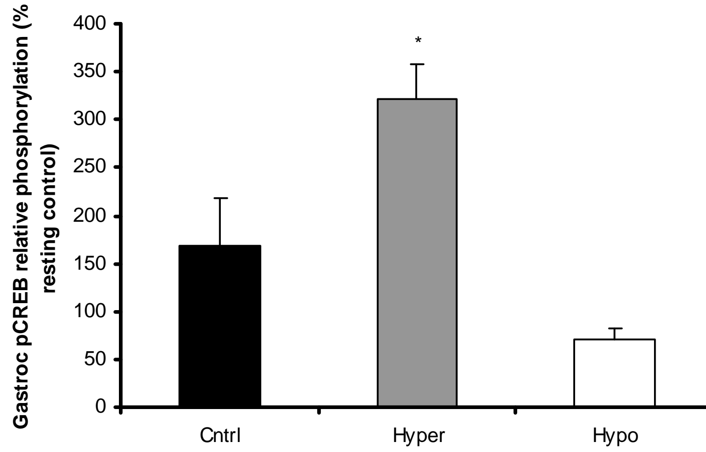
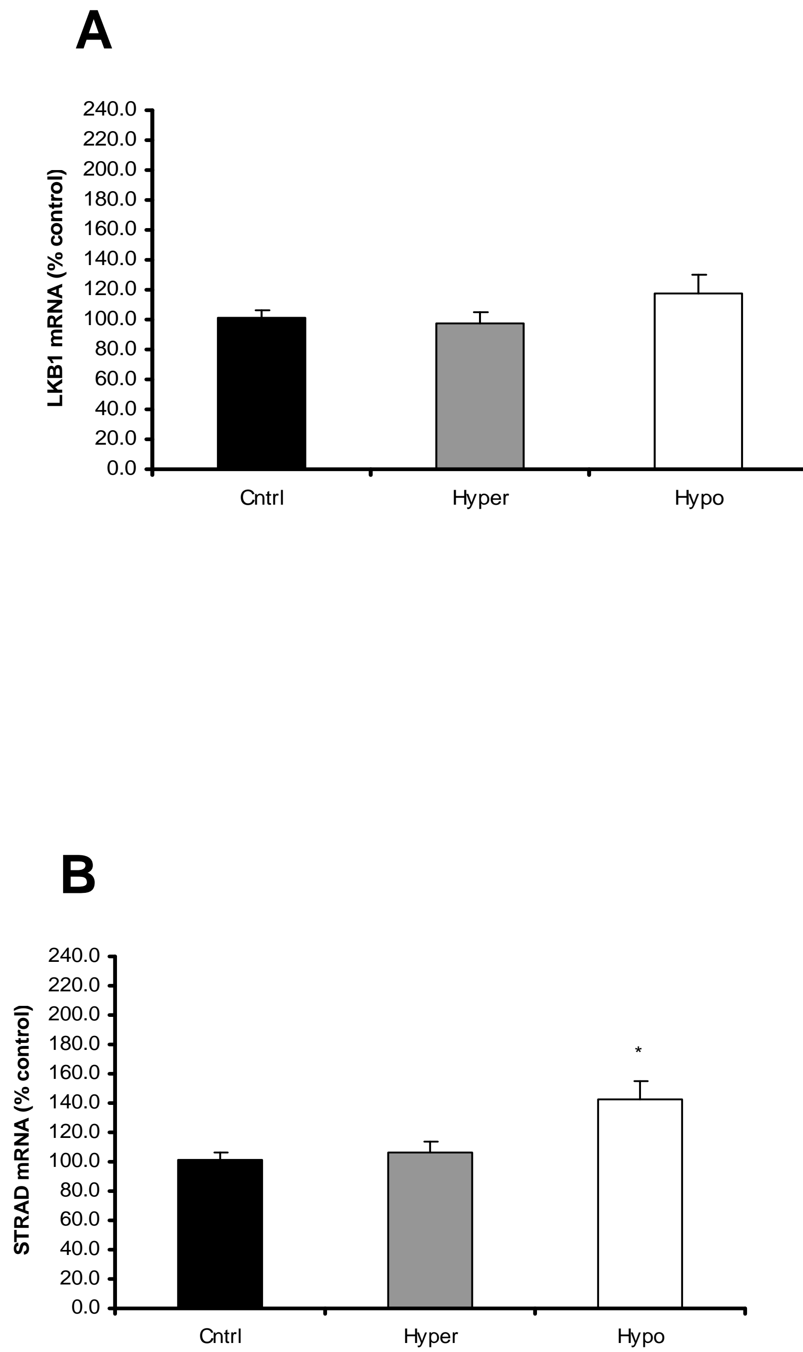

D
Gastroc pCREB relative phosphorylation (% resting control)
400
350
300
250
200
150
100
50
0
*
Cntrl
Hyper
Hypo

Figure 6.



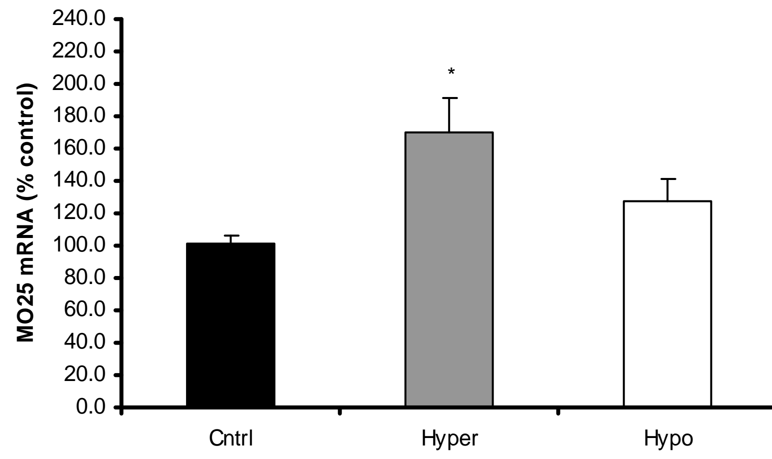

C
MO25 mRNA (% control)
240.0
220.0
200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
*
Cntrl
Hyper
Hypo

Table 1. LKB1, STRAD, and MO25 protein expression in heart and skeletal muscle.

AMPKK Complex Proteins			
	LKB1	**STRAD**	**MO25**
	Red Quadriceps		
Control	100	100	100
PTU	77±6 *	103±3	80± 11
T3&4	94±4	103±6	148±12 *,°
	White Quadriceps		
Control	100	100	100
PTU	118±8	96±3	90±5
T3&4	132±11 *	107±5 °	124±10 *,°
	Gastrocnemius		
Control	100	100	100
PTU	118±7	101±5	120±27
T3&4	125±12	102±6	208±32 *
	Heart		
Control	100	100	100
PTU	95±4	105±12	64±9 *
T3&4	100±4	82±11	140±17 *,°

Values are means ± SE (n =9). PTU, propylthiouracil; T3&4, thyroid hormone treatment for 4 wk.
* P < 0.05 vs. control. ° P < 0.05 vs. PTU.

Curriculum Vitae

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Education

M.S. Physiology and Developmental Biology from Brigham Young University, August 2007.

- Thesis: Regulation of LKB1-STRAD-MO25 Complex Expression and Activation of AMPK in Skeletal Muscle by Thyroid Hormone.
- GPA: 3.96/4.0

B.S. in Biology from Brigham Young University, *Magna Cum Laude,* April 2006

- GPA: 3.93/4.0

Special Skills

- Can speak both Italian and Spanish
- Certified Phlebotomist from Utah School of Phlebotomy, May 2004.

Work Experience

- Researcher under Dr. William Winder, May 2006 - Present.
- Chemistry Central Stockroom Clerk, May 2005 – May 2006.

Honors and Awards

- Phi Eta Sigma, Freshman Honor Society, 2001
- University Scholarship, Full Tuition for one year. 2000-2006.