Thyroid hormone effects on LKB1, MO25, phospho-AMPK, phospho-CREB, and PGC-1α in rat muscle


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Submitted 19 September 2007; accepted in final form 29 July 2008

Thyroid hormone effects on LKB1, MO25, phospho-AMPK, phospho-CREB, and PGC-1α in rat muscle. J Appl Physiol 105: 1218–1227, 2008. First published July 31, 2008; doi:10.1152/japplphysiol.00997.2007.—Expression of all of the isoforms of the subunits of AMP-activated protein kinase (AMPK) and AMPK activity is increased in skeletal muscle of hyperthyroid rats. Activity of AMPK in skeletal muscle is regulated principally by the upstream kinase, LKB1. This experiment was designed to determine whether the increase in AMPK activity is accompanied by increased expression of the LKB1, along with binding partner proteins. LKB1, MO25, and downstream targets were determined in muscle extracts in control rats, in rats given 3 mg of thyroxine and 1 mg of triiodothyronine per kilogram chow for 4 wk, and in rats given 0.01% propylthiouracil (PTU; an inhibitor of thyroid hormone synthesis) in drinking water for 4 wk (hypothyroid group). LKB1 and MO25 increased in the soleus of thyroid hormone-treated rats vs. the controls. In other muscle types, LKB1 responses were variable, but MO25 increased in all. In soleus, MO25 mRNA increased with thyroid hormone treatment, and STRAD mRNA increased with PTU treatment. Phospho-AMPK and phospho-ACC were elevated in soleus and gastrocnemius of hyperthyroid rats. Thyroid hormone treatment also increased the amount of phospho-AMPK response element binding protein (CREB) in the soleus, heart, and red quadriceps. Four proteins having CREB response elements (CRE) in promoter regions of their genes (peroxisome proliferator-activated receptor-γ coactivator-1α, uncoupling protein 3, cytochrome c, and hexokinase II) were all increased in soleus in response to thyroid hormones. These data provide evidence that thyroid hormones increase soleus muscle LKB1 and MO25 content with subsequent activation of AMPK, phosphorylation of CREB, and expression of mitochondrial protein genes having CRE in their promoters.

adenosine 5′-monophosphate-activated protein kinase; acetyl-coenzyme A carboxylase; muscle mitochondria

EXPERIMENTAL HYPERTHYROIDISM has previously been demonstrated to increase mitochondrial enzyme content of rat skeletal muscle and other tissues (48, 49). Rat soleus muscle, consisting predominantly of type I, slow-twitch fibers, is much more sensitive than other muscles to this action of thyroid hormones (3, 48), possibly due to increased thyroid hormone receptor expression (3). This model has been used to study some of the basic molecular signaling pathways responsible for this effect of thyroid hormone excess on mitochondrial biogenesis (18, 19, 33). Hyperthyroidism is one of relatively few conditions that increases metabolic rate and increases expression of mitochondrial proteins in skeletal muscle. Previous studies suggest that the LKB1/AMP-activated protein kinase (AMPK) signaling pathway is important in mitochondrial biogenesis (47, 53). In this present study, the adaptation of the LKB1/AMPK signaling pathway to hypo- and hyperthyroidism was investigated, along with downstream targets regulated by this pathway.

AMPK acts as a fuel gauge for the cell, responding to changes in cellular energy (13, 50). AMPK is activated during exercise, ischemia, hypoxia, and other forms of cellular stress (37). AMPK is inactive until phosphorylated by an upstream kinase at the threonine 172 site of the α-subunit (40, 45). Although other kinases [CaMKK-β and transforming growth factor-β activated kinase (TAK1)] can phosphorylate AMPK, a protein complex including LKB1 appears to be the major upstream kinase for AMPK in skeletal muscle (14, 20, 22, 36, 45, 47). In other cells and tissues, optimal phosphorylation/activation of AMPK by LKB1 requires association of LKB1 with STRAD and MO25 (1, 4, 15). Evidence has been presented for a role of CaMKK-β at the onset of mild tetanic contractions (20) and a role for CaMKK-α, CaMKK-β, and possibly TAK1 during overload-induced hypertrophy (28). Expression and activity of CaMKK-α and CaMKK-β, as well as phosphorylation of TAK1, were found to increase manifold in response to muscle overload (28), implying that calcium-triggered activation of AMPK may play a more important role in hypertrophied muscle.

A previous study in this laboratory (33) demonstrated that all of the subunits of AMPK were increased in skeletal muscle with thyroid hormone treatment, especially in the soleus and red quadriceps. An increase in both acetyl-CoA carboxylase (ACC) protein content and phosphorylation was also observed. At this point, however, the AMPKK complex had not yet been characterized. We, therefore, designed an experiment to test the hypothesis that thyroid hormone treatment would increase the expression of LKB1, STRAD, and MO25.

We also were interested in studying effects of novel downstream targets of the LKB1/AMPK signaling pathway that could be influenced by thyroid state. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a transcriptional regulator for genes involved in mitochondrial biogenesis, fatty acid oxidation, and gluconeogenesis (35). Skeletal muscle-specific LKB1 knockout mice show a decrease in PGC-1α, as well as mitochondrial proteins (47). Ircher et al. (18) found that PGC-1α expression increased in skeletal muscle after 5 days of thyroid hormone treatment, with the largest increase occurring in the soleus.

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Cyclic AMP response element binding protein (CREB) is a well-characterized transcription factor activated by various stimuli (39). It was originally discovered as a downstream target of the cAMP pathway. CREB is activated by phosphorylation at the serine 133 position (7, 12). CREB is involved in a variety of cellular processes, such as proliferation, development, and differentiation. It is also involved in regulation of hepatic gluconeogenesis by glucagon and epinephrine (12, 39). Recent experiments in our laboratory showed that AMPK and a variety of cellular processes, such as proliferation, development, and differentiation, are stimulated at the serine 133 position (46). The promoter region of the PGC-1α gene, as well as other thyroid hormone-responsive genes of proteins involved in ATP production, contain CREB response elements (CREs). Thus it is possible that enhanced phosphorylation of CREB by AMPK could mediate some effects of thyroid hormone on expression of these genes. We hypothesized that CREB phosphorylation would increase in skeletal muscle in thyroid hormone-treated rats.

We hypothesized that the hyperthyroid condition would result in chronic activation of AMPK due to the increase in energy demand and oxygen consumption, even at rest. The chronic AMPK activation would result in increased CREB phosphorylation and PGC-1α expression. Expression of proteins with CREs in promoters of their genes would be expected to increase. We also expected to see greater absolute AMPK activation in the hyperthyroid muscle during times of muscle contraction when energy demand is markedly increased. Under these conditions, the AMPK activity would be predicted to be higher in the hyperthyroid state due to increased expression of AMPK subunits and possibly to increased expression of components of the upstream kinase(s).

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 0600 to 1800 h. Rats weighed 200 ± 20 g at the beginning of treatments. Rats were divided into three treatment groups. The first group [control (Cntrl)] received normal rodent diet (Harlan Teklad rodent diet, Madison, WI) under 12 h of light:12 h of dark per 24 h light:dark cycle. The second group [hypothyroid (Hypo)] received normal rodent diet and 0.1% propylthiouracil (PTU) in drinking water for 4 wk for the purpose of inhibiting thyroid hormone synthesis. The third group [hyperthyroid (Hyper)] 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. These dosages have been used previously in studies on muscle mitochondrial biogenesis and are well tolerated by the rats (48, 49). On the afternoon before the rats were killed, they were given 20 g of either normal rodent diet (Cntrl and Hypo) or powdered rodent diet containing thyroid hormones (Hyper).

Muscle stimulation, collection, and homogenization. Rats were anesthetized with pentobarbital sodium (0.08 mg/g body wt) at least 30 min before beginning the procedure. The right soleus and gastrocnemius were isolated and frozen in ice-water bath and stored at −80°C for 30 min before being homogenized. The left tibial nerve was isolated and stimulated at a frequency of 1/s, 10-ms duration, 15 V, for 5 min. The left gastrocnemius and soleus were then removed and homogenized in 9 or 19 (soleus) volumes 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 homogenates were centrifuged at 1,200 g for 5–10 min at 4°C, and the supernatants were kept for analysis.

AMPK and PKA activity and LKB1 activity. The α1- and α2-AMPK activities were determined on immunoprecipitates from soleus homogenates, as described previously (32). LKB1 was immunoprecipitated from 120 μl of soleus homogenate using 4.5 μl (packed volume) Protein G Sepharose linked to 3.5 μg LKB1 antibody (sc-5640, Santa Cruz Biotechnology). Immunoprecipitates were washed twice with homogenization buffer + 0.5 M NaCl, then twice with 40 mM HEPES, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 5 mM MgCl2, and 0.8 mM EGTA (pH 7). Immunoprecipitated LKB1 activity toward LKB1 substrate (LSNL-1H4Q6KFL0TFCOG5PLYRR) was then assessed in a 50-μl reaction volume consisting of 40 mM HEPES, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl2, 0.2 mM ATP, 0.2 mM LKB1, and 5–10 μCi [γ-32P]ATP, and incubated for 15 min at 30°C with continuous shaking. After the reaction period, 40 μl of the reaction volume were spotted to PS1 papers and washed four times (15 min each) in 1% phosphoric acid. The papers were rinsed with distilled water and then acetone and dried, and the radioactivity incorporated into LKB1 substrate was assessed by scintillation counting.

Western blotting and immunodetecion. 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% [ACC, phospho (p)-ACC, PGC-1], 7.5% (p-AMPK, PGC-1, LKB1), and 10% [p-CREB, CREB, MO25, phospho LKB1] SDS-polyacrylamide gels and incubated for 15 min at 30°C with continuous shaking. After the reaction period, 40 μl of the reaction volume were spotted to PS1 papers and washed four times (15 min each) in 1% phosphoric acid. The papers were rinsed with distilled water and then acetone and dried, and the radioactivity incorporated into LKB1 was assessed by scintillation counting.

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 weighed an average of 107 ± 20 g at the beginning of treatments. Rats were divided into three treatment groups. The first group [control (Cntrl)] received normal rodent diet (Harlan Teklad rodent diet, Madison, WI) and water ad libitum. The second group [hypothyroid (Hypo)] received normal rodent diet and 0.01% propylthiouracil (PTU) in drinking water for 4 wk for the purpose of inhibiting thyroid hormone synthesis. The third group [hypothyroid (Hyper)] 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. These dosages have been used previously in studies on muscle mitochondrial biogenesis and are well tolerated by the rats (48, 49). On the afternoon before the rats were killed, they were given 20 g of either normal rodent diet (Cntrl and Hypo) or powdered rodent diet containing thyroid hormones (Hyper).

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**Comununoprecipitation of LKB1 and MO25.** Coimmunoprecipitation of MO25 and LKB1 was performed as follows. One and one-half micrograms of LKB1 antibody (sc-5640, Santa Cruz Biotechnology) were linked to 8 μl (packed volume) ExactaCruz C IP matrix (Santa Cruz Biotechnology) for 2 h at 4°C. The matrix was washed three times with ice-cold phosphate-buffered saline. Sixty microliters of clarified soleus homogenate were rotated end over end overnight with the antibody-matrix complex at 4°C. The LKB1 immunoprecipitates were then washed three times with homogenization buffer and then resuspended in 75 μl of 1 × loading buffer (with 2.5% β-mercaptoethanol). Samples were boiled for 5 min, then 25 μl of sample per well were loaded onto 7.5% (for LKB1) and 10% (for MO25) Tris-HCl gels (Bio-Rad Criterion System, Hercules, CA), and Western blotting/immunodetection was performed as described above using primary antibodies for LKB1 (1:5,000, Upstate no. 07-694) and MO25 (1:50,000, described above) and a secondary antibody (1:5,000, Rabbit ExactaCruz C-HRP, Santa Cruz Biotechnology).

**Real-time PCR.** Relative changes in mRNA with thyroid status were examined by real-time PCR. Total mRNA was isolated from 20–30 mg of tissue from Cntrl, Hyper, and Hypo 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 as follows: LKB1 forward, AGAG-GAAATGGGTTGATGAGGA; LKB1 reverse, CCGGCTTCTC-GGTTCA; MO25 forward, GTGTTGGATGAATAATCGTGT; MO25 reverse, AGCTGTTGACGGCCCTGT; STRAD forward, GTGCATGGCCGTTCTTAGTTG; and 18S reverse, GCCACCTGTCCTCCTAAGAATG. 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 obtain evidence for splice variations in mRNA from muscle, although the 55-kDa band may indeed be a covalently increased to the peak CT value. The Cycle threshold (Ct) was calculated automatically by the ABI software. Relative fold expression was calculated using the 2–ΔΔCt method after normalizing to 18S RNA (24).

**RESULTS**

**Evidence of hyper- and hypothyroid conditions.** Final body weights for both Hyper and Hypo rats were significantly less than that for the Cntrl (P < 0.05) (Table 1). As evidence that the PTU was indeed inhibiting thyroid hormone production, there was marked hyptrophosis of the thyroid in Hypo rats (Table 1). The fat pad weights for Hyper and Hypo rats were both significantly less than for the Cntrl, and the fat pad weight of Hyper rats was significantly less than that for both Cntrl and Hypo rats (P < 0.05) (Table 1). The heart weights for the Hyper rats were significantly greater (P < 0.05) than for Cntrl or Hypo rats, whether expressed as absolute weight or relative to body weight (Table 1). During the last week before the animals were killed, the average daily food intake was ∼50% greater for Hyper and 25% less for Hypo than for Cntrl rats. Plasma triiodothyronine and thyroxine concentrations were decreased markedly in Hypo rats and increased significantly in Hyper rats (Table 1).

**LKB1, STRAD, and MO25 Western blot in skeletal muscle.** LKB1 and MO25 migrated in the gel to the approximate positions based on their molecular mass (LKB1 at 55 kDa and MO25 at 40 kDa). In a previous study, our laboratory reported MO25 as a 55-kDa band on our gels (44). We attributed this higher molecular mass band to expression of a splice variant in skeletal muscle, but have subsequently been unsuccessful in obtaining evidence for splice variations in mRNA from muscle. Although the 55-kDa band may indeed be a covalently modified variant of MO25, we have reported the 40-kDa band in the present experiments. Figure 1 shows that LKB1 and MO25 were present in significantly higher amounts (150 and 160% of Cntrl values, respectively) in the Hyper rats than in Cntrl rats in soleus muscle (P < 0.05). In heart, red quadriceps, white quadriceps, and gastrocnemius muscle, MO25 expression increased to ∼140, 150, 125, and 210% of Cntrl values, respectively, in the Hyper rats (P < 0.05). LKB1 response to altered thyroid hormone status varied among the different muscle types (see Table 2). ERK1/2 were also measured to determine whether this increase in expression of the LKB1/AMPK proteins was part of a generalized increase in protein synthesis. ERK2 expression was not significantly influenced by the treatments, and ERK1 was actually decreased in Hyper soleus muscle compared with Cntrl (−44%) (data not shown).

**Immunoprecipitation studies.** Figure 2 shows that immunoprecipitated LKB1 was higher in soleus muscle of the Hyper rats compared with Cntrl, similar to the magnitude of the increase seen in Western blots of supernatants of whole homogenates. MO25 was also detected in the LKB1 immunoprecipitates. MO25 in the immunoprecipitates from Cntrl, Hyper, and Hypo rats also occurred in approximately the same ratio as was seen in the crude homogenates. Only the 40-kDa band was observed in the immunoprecipitates. LKB1 activity of immunoprecipitates showed the same pattern as the LKB1 and MO25 Western blots.

**AMPK activity, p-ACC, and p-AMPK Western blot in resting and stimulated skeletal muscle.** A previous study showed that both ACC and p-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 min. Both gastrocnemius and soleus muscles showed an increase in both p-AMPK and p-ACC in Hyper under resting conditions compared with Cntrl (see Figs. 3 and 4). In the gastrocnemius, p-AMPK increased in response to stimulation in Cntrl, Hyper, and Hypo rats. Stimulated values were not different in the three groups. However, p-ACC, the downstream target of AMPK, was higher both at rest and after 5-min stimulation in the Hyper group (Fig. 3). In the soleus, the

**Table 1. Evidence of hyper- and hypothyroidism**

<table>
<thead>
<tr>
<th></th>
<th>Cntrl</th>
<th>Hyper</th>
<th>Hypo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight, g</td>
<td>283±11</td>
<td>249±12*</td>
<td>228±8*</td>
</tr>
<tr>
<td>Thyroid weight, mg</td>
<td>19±2</td>
<td>11±1*</td>
<td>71±9*</td>
</tr>
<tr>
<td>Retroperitoneal fat pad, % body wt</td>
<td>0.58±0.04</td>
<td>0.08±0.01*</td>
<td>0.39±0.03*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>0.79±0.02</td>
<td>1.36±0.07*</td>
<td>0.58±0.02*</td>
</tr>
<tr>
<td>Heart weight, % body wt</td>
<td>0.28±0.01</td>
<td>0.55±0.02*</td>
<td>0.25±0.01</td>
</tr>
<tr>
<td>Daily food intake last week, g</td>
<td>30.7±0.4</td>
<td>40.0±1.3*</td>
<td>22.9±1.1*</td>
</tr>
<tr>
<td>Plasma triiodothyronine, nm</td>
<td>1.1±0.1</td>
<td>4.5±0.5*</td>
<td>0.1±0.06*</td>
</tr>
<tr>
<td>Plasma thyroxine, nm</td>
<td>31±3</td>
<td>60±8*</td>
<td>2±1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9. Cntrl, control rats; Hyper (hyperthyroid), rats thyroid hormone treatment for 4 wk; Hypo (hypothyroid), propylthiouracil-treated rats. *Significantly different from Cntrl, P < 0.05.
after electrical stimulation (Fig. 4). The LKB1 and MO25 expression in Hyper rats was significantly higher than in Ctrl. Values for soleus total AMPK were increased in muscle of the Hyper rats. The total ACC in soleus were 100$^\pm$7 for Ctrl, 77$^\pm$13 for Hyper, and 87$^\pm$11 for Hypo (n = 5).

PP2C Western blot. PP2C was not significantly influenced in soleus muscle by thyroid state. Values were 100$^\pm$7 for Ctrl, 77$^\pm$13 for Hyper, and 87$^\pm$11 for Hypo.

**CREB and PGC-1α Western blot in muscle.** CREB protein expression decreased by 20% in heart muscle in Hypo rats compared with the other groups. CREB did not change among the treatment groups for the other muscle types. Figure 5 shows that PGC-1α expression was 50% higher in the soleus of Hyper rats than Ctrl rats (P < 0.05), but it was not significantly changed in Hypo rats. There was no significant change in any of the other muscle types (data not shown).

**p-CREB Western blot in resting and stimulated muscle.** Under resting conditions, p-CREB levels were significantly higher in Hyper rats in the heart, red quadriceps, and soleus (70, 40, and 120% higher than Ctrl, respectively) than in the Ctrl rats (see Fig. 6). Upon electrical stimulation, however, a 50% increase occurred in soleus, and a twofold increase in CREB phosphorylation occurred in the gastrocnemius of the Hyper rats. (Fig. 7). In Ctrl and Hypo rats, there was no significant increase in p-CREB upon stimulation (Fig. 7).

Expression of proteins coded by genes having CREs in their promoters. Cytochrome c, UCP3, and hexokinase II were all significantly increased in soleus muscle in response to thyroid hormone treatment (Table 3). Each of these proteins was higher in the Hyper rats than in either the Ctrl or Hypo rats. Cytochrome c and UCP3 were significantly reduced in Hyper rats compared with Ctrl, and a trend was noted in hexokinase II. UCP3 fluctuated most markedly as a function of thyroid status.

**LKB1, STRAD, and MO25 mRNA expression.** Relative levels of LKB1, STRAD, and MO25 mRNA were measured in the heart and skeletal muscle.

<table>
<thead>
<tr>
<th>AMPKK Complex Proteins</th>
<th>LKB1</th>
<th>MO25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red quadriceps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>100$^\pm$4</td>
<td>100$^\pm$1</td>
</tr>
<tr>
<td>Hyper</td>
<td>94$^\pm$4</td>
<td>148$^\pm$12$^*$</td>
</tr>
<tr>
<td>Hypo</td>
<td>77$^\pm$6*</td>
<td>80$^\pm$11</td>
</tr>
<tr>
<td>White quadriceps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>100$^\pm$7</td>
<td>100$^\pm$8</td>
</tr>
<tr>
<td>Hyper</td>
<td>132$^\pm$11*</td>
<td>124$^\pm$10$^*$</td>
</tr>
<tr>
<td>Hypo</td>
<td>118$^\pm$8</td>
<td>90$^\pm$5</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>100$^\pm$10</td>
<td>100$^\pm$15</td>
</tr>
<tr>
<td>Hyper</td>
<td>125$^\pm$12</td>
<td>208$^\pm$32$^*$</td>
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<td>Hypo</td>
<td>118$^\pm$7</td>
<td>120$^\pm$27</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>100$^\pm$4</td>
<td>100$^\pm$9</td>
</tr>
<tr>
<td>Hyper</td>
<td>100$^\pm$4</td>
<td>140$^\pm$17$^*$</td>
</tr>
<tr>
<td>Hypo</td>
<td>95$^\pm$4</td>
<td>64$^\pm$9</td>
</tr>
</tbody>
</table>

Values are relative intensities of chemoluminescence in respective bands compared with controls and are means $^\pm$ SE; n = 9. AMPKK, AMP-activated protein kinase kinase. Proteins were determined by Western blotting of homogenates from the respective muscles using antibodies, as indicated in MATERIALS AND METHODS. $^*$P < 0.05 vs. Ctrl. $^*$P < 0.05 vs. Hypo.

Fig. 1. Relative expression of soleus muscle LKB1 and MO25 in control (Ctrl), thyroid hormone-treated [hyperthyroid (Hyper)], and propylthiouracil (PTU)-treated [hypothyroid (Hypo)] rats, determined by Western blot. LKB1 expression in Hyper and Hypo rats was significantly different from Ctrl, and MO25 expression in Hyper rats was significantly higher than in Ctrl. Values are means $^\pm$ SE; n = 9. $^*$Significantly different from control, P < 0.05. α$_2$-AMPK activity was higher in the Hyper group at rest and after electrical stimulation (Fig. 4). The α$_2$-AMPK activity was not significantly different at rest but was higher in the Hyper group after stimulation. Although the AMPK activity and p-AMPK in soleus of Ctrl rats were not influenced by electrical stimulation, p-ACC was increased, indicating the p-AMPK fraction in the contracting muscle was probably allosterically activated by the increase in AMP.

The total α-subunit content and total ACC measured by Western blot were increased in muscle of the Hyper rats. Relative values for soleus total AMPK-α were 100$^\pm$6 for Ctrl, 132$^\pm$6 for Hyper (significant difference from both groups, P < 0.05), and 95$^\pm$6 for Hypo. Relative values for total ACC in soleus were 100$^\pm$10 for Ctrl, 180$^\pm$12 for Hyper (significant difference from both groups, P < 0.05), and 79$^\pm$14 for Hypo. For gastrocnemius, total AMPK-α was 100$^\pm$5 for Ctrl, 131$^\pm$9 for Hyper (significant difference from both groups, P < 0.05), and 90$^\pm$5 for Hypo. Relative values for total ACC in gastrocnemius were 100$^\pm$10 for Ctrl, 124$^\pm$7 for Hyper (significant difference from the Hyper group only, P < 0.05), and 89$^\pm$10 for Hypo.

In the heart, relative values of p-AMPK were 100$^\pm$11, 140$^\pm$12, and 64$^\pm$6 for Ctrl, Hyper, and Hypo rats, respectively. The Ctrl values may have been inordinately high due to the fact that the heart was removed last, shortly after blood collection and was, therefore, likely hypoxic at the time of collection. Nevertheless, statistically significant differences were noted between all treatment groups (P < 0.05).

PP2C Western blot. PP2C was not significantly influenced in soleus muscle by thyroid state. Values were 100$^\pm$7 for Ctrl, 77$^\pm$13 for Hyper, and 87$^\pm$11 for Hypo (n = 5).

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soleus of Cntrl, Hypo, and Hyper rats. LKB1 mRNA levels did not change with thyroid hormone or PTU treatment (data not shown). STRAD mRNA did not increase with thyroid hormone treatment, but, surprisingly, it increased to 145% of the Cntrl with PTU treatment ($P < 0.05$). MO25 mRNA was 165% of the Cntrl with thyroid hormone treatment ($P < 0.05$).

**DISCUSSION**

A previous study from this laboratory (33) 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. The elevated AMPK phosphorylation in the Hyper rats was likely due in part to the increased expression of AMPK subunits, as shown in the present study and in our laboratory’s previous study (33). LKB1 and associating binding partner proteins were not characterized as an AMPKK until 2003 (4, 15), and it was, therefore, impossible at the time of the study by Park et al. (33) 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, similar to the increase in AMPK subunits. This proved to be the case, although the increase in LKB1 and MO25 seemed more tissue dependent than the changes in AMPK subunit expression. LKB1 and MO25 increased significantly in the soleus of Hyper rats. MO25 increased significantly in all other tissue types analyzed: heart, gastrocnemius, red quadriceps, and white quadriceps. This variable response between different muscle types may relate to the higher thyroid hormone receptor content of soleus.

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**Fig. 2.** Western blots of LKB1 and MO25, and LKB1 activity in LKB1 immunoprecipitates (IP) from soleus muscles of Cntrl, Hyper, and Hypo rats. Values are means ± SE; $n = 5–7$. *Significantly different from control, $P < 0.05$. Due to interassay variation, LKB1 activity is expressed as percentage of control. The average LKB1 activity in soleus muscles from Cntrl rats was 4.0 ± 1.2 pmol·g$^{-1}$·min$^{-1}$.

**Fig. 3.** Effect of 5-min electrical stimulation (Stim) on increase in phosphorylation of acetyl-CoA carboxylase (p-ACC) and on phospho-AMP-activated protein kinase (p-AMPK) in gastrocnemius of Cntrl, Hyper, and Hypo rats. Values are means ± SE; $n = 7–9$. *Significantly different from resting muscle of the same treatment, $P < 0.05$. †Significantly different from Cntrl rest or Cntrl Stim, $P < 0.05$. 

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1222 THYROID HORMONE EFFECTS ON LKB1, PGC-1α, AND p-CREB IN MUSCLE

*J Appl Physiol* • VOL 105 • OCTOBER 2008 • www.jap.org

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muscle (3). The absence of a decrease in LKB1 protein and activity in immunoprecipitates of the soleus of Hypo rats may be explained by a delay in decline of plasma thyroid hormones after PTU administration was initiated. A small decrease was noted in the soleus homogenate supernatant blots, and a trend was noted in the immunoprecipitates. PTU inhibits iodination of tyrosine residues of thyroglobulin and coupling of iodotyrosines to form thyroxine (5). The thyroid normally stores considerable thyroxine in peptide linkages of thyroglobulin in the follicles, and it was likely several days after initiation of PTU treatment before a significant drop in plasma levels of thyroid hormones occurred. With a more prolonged treatment with PTU, larger decreases in LKB1 would likely be observed.

Plasma adipokines have previously been demonstrated to influence both expression and activity of AMPK (41, 54). Leptin and adiponectin increase and resistin decreases activation. Prolonged leptin infusion into rats increases expression of AMPK subunits, as well as increases phosphorylation/activation (41). With the marked decrease in adipose tissue mass in the Hyper rats, we might expect to see alterations in adipokine production. Previous reports indicate plasma leptin decreases, whereas plasma adiponectin increases with hyperthyroidism (2, 6). This divergence in plasma concentration of these two activators makes it difficult to predict the net effect of excess thyroid hormones on AMPK expression and activity.

The communoprecipitation studies demonstrate association of MO25 with LKB1 in the skeletal muscle homogenates. To our knowledge, this is the first report providing evidence of association of MO25 with LKB1 in skeletal muscle. The same magnitude of increase in both LKB1 and MO25 in response to hyperthyroidism was observed in supernatants of the crude homogenates and in the LKB1 immunoprecipitate of soleus muscle. We are not aware of data clearly demonstrating that STRAD is the protein associ-

Fig. 4. AMPK activity (n = 5) of IPs and Western blots of p-AMPK (phospho-threonine-172) and p-ACC (n = 7–9) in resting and electrically stimulated soleus muscles. Values are means ± SE. *Significantly different from resting muscle of the same treatment group, †Significantly different from Cntrl rest or Cntrl Stim, P < 0.05.
ating with LKB1 in skeletal muscle. We had a STRAD antibody commercially prepared using recombinant human STRAD. This antibody detected the recombinant STRAD and a protein band in muscle extracts at the correct molecular mass. We were unable to detect STRAD in LKB1 immunoprecipitates with this antibody. It is unclear whether this was due to nonspecificity of the antibody or to the existence of another STRAD-like protein in muscle required for enhancement of LKB1 activity. Additional studies are required to show which protein binding partners are necessary for full activity of LKB1 in skeletal muscle.

The mechanism by which LKB1 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 that encode for LKB1 and MO25. This assumption proved to be incorrect. MO25 mRNA increased to 170% of Cntrl levels in response to thyroid hormone treatment, following the trend observed by Western blot, but LKB1 and STRAD mRNA did not increase significantly. STRAD (National Center for Biotechnology Information (NCBI) Accession no. AC015651) has a potential thyroid response element (GGATCACCTGAGGTCA) ~1,500 bases upstream from the start sequence, but promotors of LKB1 (STK11, NCBI Accession no. AC011544) (8, 16) and MO25 (NCBI Accession no. AC084031) were replete of thyroid response elements. It is interesting that MO25 mRNA was the only one of the three analyzed that increased, although the protein content of LKB1 and MO25 both increased in the soleus of Hyper rats. We observed similar results in our laboratory in a study on endurance training, when we analyzed the red quadriceps of trained rats (44). LKB1 and MO25 protein content of muscle increased, but only MO25 mRNA expression increased. The increase in MO25 mRNA observed in our current study may, in part, be responsible for the increase in MO25 protein expression, but it is unlikely that an increase in transcription rate is responsible for the increase in LKB1 protein content of the muscle.

The prevailing p-AMPK and p-ACC were consistently higher in resting gastrocnemius and soleus muscles, and α2-AMPK activity was higher in the soleus of the Hyper rats. The p-AMPK, but not p-ACC, was lower in the Hypo rats than in Cntrl. This chronic activation of AMPK in the Hyper rats with consequent increased phosphorylation of downstream targets could be responsible, in part, for the increase in mitochondrial biogenesis. Electrical stimulation of the left tibial nerve activated AMPK and increased phosphorylation of AMPK and

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**Fig. 5.** Relative expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) in soleus homogenates of Cntrl, Hyper, and Hypo rats was determined by Western blot. Values are means ± SE; n = 9. *Significantly different from control, P < 0.05.

**Fig. 6.** p-cAMP response element binding protein (p-CREB) (Ser 133) in heart, red quadriceps (Quad), and soleus muscles of Cntrl, Hyper, and Hypo rats. Values are means ± SE; n = 9. *Significantly different from control, P < 0.05.
CREB is a transcription factor involved in numerous cellular and metabolic processes (27). It binds to CRE on promoters of target genes, and it can be activated by phosphorylation at the serine 133 position (21). This phosphorylation allows for recruitment of CREB binding protein or p300, two similar proteins that have histone acetylase activity, as well as the ability to interact with basal transcription factors, such as TFIIB and TATA-binding protein (21, 38). There are CREs on many different genes involved in cell proliferation, differentiation, and division, as well as metabolic regulation, and, when CREB is activated, it can facilitate the transcription of these genes (30, 38, 39, 43). We observed an increase in p-CREB in the soleus, heart, and red quadriceps of Hyper rats. This increase could occur through various different pathways, since CREB can be phosphorylated by a number of different kinases (21). The best known pathway is through PKA, but it is dependent on cAMP concentration. In cultured avian myoblasts, triiodothyronine has been reported to increase cAMP and phosphorylation of CREB (25). In mature rat soleus muscle, however, cAMP levels have been shown to decrease in response to the same thyroid hormone dose used in the present study (48). It is unlikely, therefore, that the increase in CREB phosphorylation was due to PKA activation, and we must consider other kinases. Since AMPK is more active in the Hyper muscle, and CREB is a demonstrated direct target of AMPK (46), it is reasonable to suggest that AMPK mediates this increase in p-CREB. In cardiac myocytes, phosphorylation of CREB has been associated with phenylephrine-induced hypertrophy (26). Hypertrophy of cardiac myocytes does not occur in the absence of p300 and CREB binding protein signaling, along with accompanying histone acetylase activity (9, 10). However, chronic activation of AMPK in cardiac myocytes after transfection with LKB1, STRAD-α, and MO25-α actually decreases phenylephrine-induced protein synthesis (31).

The increase in PGC-1α in soleus muscle of Hyper rats confirmed previous observations by Irrcher et al. (18). 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 is likely a consequence. It was interesting to note, however, that, although p-AMPK and p-CREB increased in various tissue types (albeit to a smaller extent) upon treatment with thyroid hormone, PGC-1α only increased in the soleus. This observation is consistent with the previous observation that soleus muscle (composed of type I

![Graph](https://via.placeholder.com/150)

**Fig. 7.** Effect of 5-min electrical stimulation on p-CREB (Ser 133) in soleus and gastrocnemius muscle of Cntrl, Hyper, and Hypo rats. Values are means ± SE; n = 5 for soleus and 9 for gastrocnemius. *Significantly different from control, P < 0.05. Hypo significantly different from Hyper in both muscles, P < 0.05.

ACC in the gastrocnemius of Cntrl, Hyper, and Hypo rats. After 5-min stimulation, p-ACC, but not p-AMPK, increased to higher levels in the Hyper than in the Hypo or Cntrl rats. Considering p-ACC as an endogenous reporter for AMPK activity, this provides evidence that the AMPK activity in the live working muscle was higher in the Hyper group, likely due to allosteric activation by AMP. In the soleus, electrical stimulation caused an increase in both α1- and α2-AMPK activity in immunoprecipitates, but only in the Hyper rats. The p-AMPK was increased in both Hyper and Hypo rats, and the p-ACC was increased in all three treatment groups in response to stimulation. Although no change was noted in AMPK activity or p-AMPK in the Cntrl soleus muscles in response to stimulation, the increase in p-ACC lends support to the idea that AMPK was more active in these muscles in vivo. Allosteric effects would not be detected in either the AMPK activity assay or in the Western blot for p-AMPK. It seems clear that, whether at rest or in more energy-challenging states, AMPK is more active in the Hyper muscle. This could be due to an increase in LKB1 expression and activity and to increased expression of AMPK subunits in the case of the soleus. In fact, the magnitude of the increase in p-AMPK was similar to the magnitude of the increase in total AMPK-α, implying that the same proportion of total AMPK was phosphorylated in both Cntrl and Hyper soleus. In the case of the gastrocnemius, which exhibited no significant increase in LKB1, the increased expression of AMPK subunits likely played a role.

**Table 3.** Soleus muscle expression of three proteins coded by genes with cAMP response element binding protein response elements in their promoters

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cntrl</th>
<th>Hyper</th>
<th>Hypo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase II</td>
<td>100±11</td>
<td>160±7*</td>
<td>70±7†</td>
</tr>
<tr>
<td>UCP-3</td>
<td>100±26</td>
<td>600±86*</td>
<td>5±1†</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>100±5</td>
<td>129±9*</td>
<td>86±4†</td>
</tr>
</tbody>
</table>

Values indicate intensity of the bands relative to bands from muscles of control rats and are means ± SE; n = 9. The proteins were determined by Western blotting of soleus homogenates using antibodies to the respective proteins, as indicated in MATERIALS AND METHODS. UCP-3, uncoupling protein 3. *Significantly different from control, P > 0.05. †Significantly different from hyperthyroid, P < 0.05.
fibrils) is more responsive than other muscle types (type II) to thyroid hormone induction of mitochondrial biogenesis (48, 52).

The increase in LKB1 and AMPK signaling induced 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 (34, 42, 48). The induction of PGC-1α has been shown in muscle cells in culture to induce synthesis of mitochondrial enzymes involved in oxidation of pyruvate and fatty acids (11, 23, 29). AMPK has been shown to relieve the malonyl-CoA inhibition of carnitine palmitoyltransferase-1 by inactivating ACC (51). It, therefore, seems possible that the increase in fatty acid oxidation observed in hyperthyroid patients occurs by increasing expression of LKB1, MO25, and AMPK subunits, leading to subsequent AMPK activation and consequent increased PGC-1α expression. It has been shown that thyrotoxicosis induces increased expression of mitochondrial proteins, such as citrate synthase, cytochrome oxidase, α-glycerophosphate dehydrogenase, and 3-hydroxybutyrate dehydrogenase, in skeletal muscle, especially the soleus (48, 52). Malate dehydrogenase, succinate dehydrogenase, citrate synthase activity, and hexokinase II all increase in soleus of rats treated with 5-aminomimidazole-4-carboxamide-1-β-n-ribofuranoside injections (53). Conversely, LKB1 knockout mice exhibit decreases in citrate synthase activity (47). It is, therefore, conceivable that thyroid hormone-induced increases in LKB1 complex proteins, and the ensuing activation of AMPK, mediate, in part, the thyroid hormone-induced increase in oxidative capacity.

Although most of the actions of thyroid hormones are mediated by changes in gene transcription, evidence has recently been provided indicating triiodothyronine can activate AMPK acutely in soleus, plantaris, and heart within 2 h of injection (19). Authors point out, however, that plasma levels of triiodothyronine used to elicit this response were considerably higher than the physiological range. The mechanism of this rapid activation and the dose response for these acute effects are yet to be determined.

In conclusion, we found that thyroid hormone treatment does increase LKB1 and MO25 protein content of soleus muscle, as well as the phosphorylation of AMPK upon electrical stimulation. LKB1 and MO25 are binding partners in skeletal muscle and increase concurrently in soleus muscle in response to thyroid hormone. Thyroid hormone treatment also increases the phosphorylation of CREB in the soleus and increases muscle content of four proteins that have CREs. These results suggest that thyroid hormones play a role in the regulation of the LKB1/AMPK signaling proteins and on the activation of CREB in skeletal muscle.

REFERENCES


THYROID HORMONE EFFECTS ON LKB1, PGC-1α, AND p-CREB IN MUSCLE


