Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle


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Received 29 January 2002; accepted in final form 16 February 2002

Park, S. H., S. R. Gammon, J. D. Knippers, S. R. Paulsen, D. S. Rubink, and W. W. Winder. Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle. J Appl Physiol 92: 2475–2482, 2002; 10.1152/japplphysiol.00071.2002.—AMP-activated protein kinase (AMPK) is activated during muscle contraction in response to the increase in AMP and decrease in phosphocreatine (PCr). Once activated, AMPK has been proposed to phosphorylate a number of targets, resulting in increases in glucose transport, fatty acid oxidation, and gene transcription. Although it has been possible to directly observe phosphorylation of one of these targets, acetyl-CoA carboxylase (ACC) in vitro, it has been more difficult to obtain direct evidence of ACC phosphorylation in contracting skeletal muscle. In these experiments using a phosphoserine antibody to ACC and a phosphothreonine antibody to AMPK, evidence was obtained for phosphorylation and activation of ACC in vitro, in gastrocnemius muscle electrically stimulated at different frequencies, and in muscle from rats running on the treadmill. Significant negative linear correlations between phosphorylation of ACC and AMPK activity were observed in all models (P < 0.01). The decline in ACC activity was related to the decrease in PCr and the rise in AMP. A relationship between phosphorylation of AMPK (threonine 172) and activity of AMPK immunoprecipitated with anti-α2 subunit antibody preparation was also observed. These data provide the first evidence of a direct link between extent of phosphorylation of these proteins at sites recognized by the antibodies and activity of the enzymes in electrically stimulated muscle and in muscle of rats running on the treadmill.

creatine; fatty acid oxidation; malonyl-CoA; palmitoyl-carnitine transferase; phosphocreatine; AMP-activated protein kinase

MALONYL-COENZYME A (CoA) inhibits carnitine palmitoyltransferase 1 (CPT1), a rate-limiting enzyme of fatty acid oxidation, in skeletal muscle, heart, and liver (16, 22–26, 41, 42, 50, 52). Studies in rats demonstrate that a decrease in malonyl-CoA in muscle in response to contraction removes inhibition of CPT1 and allows fatty acid oxidation to increase to meet the increased energy requirement of the working muscle (24–28, 50–54). Evidence has been presented indicating that glycerol-3-phosphate acyltransferase is phosphorylated by AMP-activated protein kinase (AMPK), resulting in inactivation and shunting of fatty acids toward oxidation and away from the triacylglycerol synthesis pathway in muscle (30). The activity of acetyl-CoA carboxylase, the citrate-activated enzyme that synthesizes malonyl-CoA, decreases in muscle during exercise or in response to muscle contraction (7, 20, 37–40, 50, 52). Purified muscle acetyl-CoA carboxylase can be phosphorylated in vitro by AMP-activated protein kinase, with a consequent decrease in activity of the enzyme (53). The citrate activation curve is shifted to the right, resulting in a marked decrease in activity in the physiological range of citrate concentrations (53). These same changes in kinetic properties of ACC are observed in muscle during exercise (20, 37, 38, 46, 53).

Abundant data are now available indicating that AMPK is activated in response to muscle contraction (8, 11, 20, 31, 37, 38, 46, 50–53, 57). Once activated, AMPK has been proposed to phosphorylate a number of targets in muscle involved in ATP production, resulting in increases in glucose transport, fatty acid oxidation, and gene transcription (16, 29, 39, 50, 52). AMPK is a heterotrimeric protein kinase that is activated by increases in muscle AMP, an allosteric activator, and by decreases in muscle phosphocreatine (PCr), an allosteric inhibitor (3, 5, 6, 16, 21, 36, 47). Studies have also demonstrated that AMPK can be phosphorylated on threonine 172 of the α-subunit, resulting in activation (6, 14, 16, 18, 43). Until recently, it was not possible, however, to obtain a direct quantification of the extent of phosphorylation of muscle ACC by AMPK in exercising or electrically stimulated muscle. Phosphoserine antibodies are now available for assessing specific phosphorylation of skeletal muscle acetyl-CoA carboxylase (ACC) at the activity-modulating AMPK target site (the site equivalent to serine 79 of liver ACC). In addition, phosphothreonine antibodies are available for quantitation of phosphorylation of threonine 172 of the α-subunit of AMPK by the upstream kinase, AMPK kinase. Interesting data have already been reported indicating phosphorylation of ACC in contracting muscle of exercising human subjects (4, 44). The importance of study of regulation of ACC in muscle was recently highlighted in a report.

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indicating that knockout mice lacking the muscle iso-
form of ACC show less fat accumulation than do nor-
mal mice and higher rates of fatty acid oxidation in
isolated muscle compared with those of control mice
(1). It has also been suggested that dysregulation of
fatty acid oxidation could contribute to development of
insulin insensitivity and Type 2 diabetes (24). Rela-
tively little information is available regarding the re-
lationships among AMPK activity, ACC activity, and
the extent of phosphorylation of these proteins. In the
present studies, a wide range of phosphorylation states
were generated by using purified AMPK and ACC, by
using electrically stimulated muscle, and by using muscle from rats run on the treadmill, allowing corre-
lation of phosphorylation state with activities of mus-
cle ACC and AMPK.

MATERIALS AND METHODS

In vitro phosphorylation of purified ACC by AMPK. ACC
was isolated from quadriceps and gastrocnemius muscles of
the rat hindlimb as described previously (53). AMPK isolated
from rat liver to the gel filtration step was obtained from the
laboratory of Dr. Grahame Hardie (Dundee, Scotland) (3).
For in vitro phosphorylation studies, ACC was precipitated
in the presence of globulin-free albumin as described previ-
ously (53, 55). Final concentrations in the reaction mix were
34 mM HEPES, 68 mM NaCl, 0.68 mM EDTA, 0.68 mM
EGTA, 0.68 mM dithiothreitol, 6.8% glycerol, 0.2 mM AMP,
and 0.12 mM ATP, pH 7.0. Purified ACC was incubated for 30
min in the absence of kinase or in the presence of 5 U/ml
AMPK or 6 U/ml cAMP-dependent protein kinase (PKA;
Sigma Chemical). Aliquots of the mixture were added to
Laemmli’s buffer (1 vol reaction mixture:2 vol water:1 vol
Laemmli’s buffer) for PAGE with the Bio-Rad Mini-Protean
II dual slab vertical electrophoresis system using Mini-Pro-
tein II 5% precast gels (Bio-Rad, Richmond, CA). Gels were
run in the presence of 0.1% SDS, 25 mM Tris, and 192 mM
glycine, pH 8.3, at 200 V for 45 min. Proteins were trans-
ferred by electroblotting from the gel to nitrocellulose mem-
brane at 100 V for 50 min. Membranes were blocked in 5%
nonfat dried milk (Bio-Rad) in PBST (139 mM NaCl, 2.7 mM
KH2PO4, 9.9 mM Na2HPO4, and 0.1% Tween 20) and then
labeled overnight with immunoaffinity-purified rabbit anti-
phospho-ACC antibody (Immunogen = synthetic peptide cor-
responding to amino acids 73–85 of rat ACC, CHMRSS[ps]
GLHLVK, conjugated to keyhole limpet hemocyanin; Up-
state Biotechnology, Waltham, MA) at a dilution of 1:2,000.
The next day, membranes were washed twice in PBST and
then in PBS (139 mM NaCl, 2.7 mM KH2PO4, 9.9 mM
Na2HPO4). Membranes were then exposed to horseradish
peroxidase-conjugated donkey anti-rabbit IgG (Amersham
Biosciences, Piscataway, NJ) for 1 h at room temperature
followed by washing twice in PBST and twice in PBS. The
phosphorylated ACC spots were then visualized on enhanced
chemiluminescence hyperfilm (Amersham Biosciences). Rel-
ative amounts of phospho-ACC were quantified by use of a
Hewlett-Packard Scan Jet 6200C and SigmaGel software
(SPSS, Chicago, IL). In a time course experiment, ACC activity
was measured as described previously (53) at 0.5 mM
citrate on aliquots removed from the phosphorylation reac-
tion mixture at intervals after addition of the AMPK. At the
same time intervals, aliquots were removed and added to the
Laemmli’s buffer mixture and frozen in liquid nitrogen.
These samples were later analyzed for ACC phosphorylation
by the Western blotting procedure described above. This
allowed generation of a wide range of ACC activities and of
ACC phosphorylation states. Samples from all time points
were run on the same gel and blot. After densitometric
scanning and quantitation, intensities of all spots were ex-
pressed relative to the darkest phosho-ACC spot on the blot.
The ACC activities and phosphorylation states were then
subjected to correlation analysis and linear regression by use
of the Number Cruncher Statistical Software (NCSS, Kaysville,
UT).

In situ stimulation of the gastrocnemius muscle. Male
Sprague-Dawley strain rats (Sasco, Wilmington, MA) were
housed in single cages in a room lighted between 6 AM and 6
PM. Rats were provided with water and Harlan Teklad rat
chow ad libitum until the time of killing. All procedures
involving use of rats were approved by the Institutional
Animal Care and Use Committee at Brigham Young Univer-
sity. On the day of the experiment, rats (age ~2 mo, body
wt = 238 ± 6 g) were anesthetized with pentobarbital sodium
(50 mg/kg body wt ip). They were kept anesthetized at a
surgical level by injection of additional anesthetic for at least
45 min before surgery. The purpose of this delay was to allow
any increase in AMPK due to the handling procedure to
return to baseline values before the beginning of the exper-
iment. The tibial nerve was then exposed by blunt dissection.
Gastrocnemius muscles were collected at rest or after stim-
ulation via the tibial nerve with single pulses of 10 ms
duration and 10 V for 5 min at frequencies of 0.2, 1, and 5 s–1.
The purpose of this procedure was to generate conditions of
a broad spectrum of AMPK activities, phosphorylated ACC,
and ACC activities. At the end of the stimulation, the gas-
trocnemius was clamp frozen between stainless steel clamps
at liquid nitrogen temperature and then stored at −90°C
until analyzed.

These muscles were ground to powder under liquid nitro-
egen and homogenized in a buffer (1 g muscle powder + 9 ml
buffer) containing 100 mM mannitol, 50 mM NaF, 100 mM
Tris, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.5, and
proteolytic enzyme inhibitors (8 trypsin inactivating units/l
aprotinin, 1 mg/l leupeptin, and 1 mg/l antipyrasin). After
centrifugation at 48,000 g for 30 min, the supernatant was
analyzed for phospho-ACC by Western blotting as described
above. ACC for citrate-dependent activity was isolated from
this homogenate as described previously. ACC activity was
determined at citrate concentrations ranging from 0 to 20
mM as described previously (53). The Grafit program (Sigma
Chemical) was used for analyzing the data to obtain the
maximum activity constant (Ks) and maximal activity as a
function of citrate (Vmax). A second homogenate (1.9) was also
prepared in 50 mM Tris·HCl, 250 mM mannitol, 50 mM NaF,
5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, pH 7.4, and proteolytic enzyme inhibitors (1
mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride,
and 5 μg/ml soybean trypsin inhibitor). The Western blot for
phospho-AMPK was performed on the 700-g supernatant of
this homogenate. The immunoaffinity-purified phospho-
AMPK primary antibody (Immunogen = synthetic phospho-
threonine peptide corresponding to residues surrounding
threonine 172, SDGELFR[Y]SICGSPNY, of the α-subunit of
human AMPK conjugated to keyhole limpet hemocyanin) was
obtained from Cell Signaling Technology (Beverly, MA).
The dilution for the Western blot was 1:1,000. Samples from
each stimulation frequency were run on the same gel and blot.
After densitometric scanning and quantitation, intensi-
ties of all spots were expressed relative to the darkest spot
on the blot.

The α1 and α2 AMPK activities were determined on immu-
noprecipitates by using commercially prepared (Affinity

J Appl Physiol • VOL 92 • JUNE 2002 • www.jap.org
Bioreagents, Golden, CO) affinity-purified antibodies to the peptides TSPDFSFLDDHHLTR (α1) and MDDSAMHIP-PCLKPH (α2) conjugated to keyhole limpet hemocyanin at the NH2 terminus via a cysteine residue. The immunoprecipitation and AMPK activity measurements were done by methods described by Hardie et al. (17), with the exception that the immunoprecipitation was overnight and the AMPK assay was on resuspended immunoprecipitate in the medium described previously (53).

A perchloric acid extract (100 mg powder/ml 6% perchloric acid) was also made of the frozen muscle powder for determination of creatine (48), PCr (19), ATP (19), lactate (12), and estimated free AMP (9). Glycogen was determined by the method of Passonneau and Lowry (35).

Effect of treadmill running on phoso-ACC in different types of muscle. Rats were run on a rodent treadmill for 5–10 min a day for 1 wk at speeds ranging from 15 to 31 m/min to accustom them to treadmill running. A jugular catheter was installed 3 days before the day of killing. On the day of the experiment, rats (age = 2 mo, body wt = 245 ± 2 g) were killed at rest or after 10 min of running at 16 m/min or after 5 min at 16 m/min + 5 min at 31 m/min up a 15% grade. They were rapidly anesthetized via the jugular catheter (35 mg pentobarbital sodium/kg body wt) at the end of the run. With intravenous administration of the anesthetic, rats are anesthetized immediately, allowing removal of the muscles in time to preserve changes in ACC and AMPK, resulting from the exercise. Although it is possible that the anesthesia procedure could alter detected responses, it is clear from recovery studies that contraction-induced AMPK and ACC activity changes are preserved for several minutes into the postexercise period (37). Muscles were removed rapidly and frozen with use of stainless steel clamps at liquid nitrogen temperature. Phospho-ACC was determined by Western blot. ACC was partially purified by use of ammonium sulfate precipitation, and activity was determined as described previously (53). This allowed correlation of phospho-ACC with ACC activity. Total AMPK activity was also determined on this resuspended ammonium sulfate precipitate (53). This method results in lower activities than the immunoprecipitation method and it does not distinguish the different isoforms, but has been used extensively to characterize this signaling system. It is not clear whether the difference in magnitude of activities between the two approaches is a result of a difference in yield or of a difference in incubation conditions.

Statistical analyses. Linear regression, correlation analysis, and analysis of variance followed by Fisher’s least significant differences tests were run by use of the Number Cruncher Statistical Software. Where appropriate, 95% confidence intervals are shown on the graphs. A probability value of 0.05 was used for all analyses for determination of statistically significant changes in response to treatments.

RESULTS

In vitro phosphorylation of muscle ACC by AMPK. Figure 1 demonstrates that the phospho-ACC antibody reacts with AMPK-phosphorylated ACC but not to any great extent with the nonphosphorylated ACC isolated from skeletal muscle. It also demonstrates that the phosphorylation is specific for the AMPK site in that phosphorylation by PKA (previously shown to phosphorylate the muscle isoform of ACC) does not result in an increase in immunoreactivity as detected by the Western blot using the phospho-ACC antibody. Figure 2, top, shows the time course of the increase in phosphorylation of ACC along with the decrease in ACC activity at 0.5 mM citrate. Figure 2, bottom, demonstrates the correlation (with 95% confidence intervals) and linear regression between ACC phosphorylation and AMPK activity.

In situ stimulation of the gastrocnemius muscle. Figure 3 demonstrates the effect of different rates of stimulation (5 min) on glycogen, lactate, adenine nucleotides, PCr, creatine, AMPK (α2-isofrom) activity, and ACC activity at 0.5 mM citrate. As can be seen from the figure, with the exceptions of PCr and ACC activity, no marked changes occurred at the lowest stimulation rate (0.2 s−1). Higher stimulation rates were accompanied by significant declines in glycogen, ATP, and PCr and significant increases in lactate, creatine, estimated free AMP, and α2 AMPK activity. The decline in ACC activity at 0.5 mM citrate appeared to occur beginning at a stimulation rate of 0.2 s−1, yet AMPK activity was significantly increased only at higher stimulation rates (1 and 5 s−1). When the relationships are examined as a function of stimulation rate, the decrease in ACC activity at 0.5 mM citrate appeared to correlate better with the decline in PCr than with the increase in AMPK activity (i.e., due to phosphorylation). The α1-isoform AMPK activity showed a similar pattern to α2 but with a smaller magnitude of change in response to stimulation. Values were 0.72 ± 0.09 for resting muscle and 1.33 ± 0.32, 1.49 ± 0.27, and 1.71 ± 0.25 nmol·g−1·min−1 for muscles stimulated at frequencies of 0.2, 1, and 5 s−1, respectively (n = 5).

Figure 4 shows the progressive inactivation of ACC at increasing rates of stimulation over the range of citrate concentrations between 0 and 20 mM. Much larger relative changes were seen at citrate concentrations near the physiological range in response to phosphorylation by AMPK. Table 1 demonstrates changes in ACC Vmax as a function of citrate as well as the Ka for citrate. Significant differences were observed at stimulation rates of 1 and 5 s−1 for both Ka and Vmax.
compared with resting values. At a stimulation rate of 0.2 s⁻¹, only $V_{\text{max}}$ was found to be significantly reduced ($P < 0.05$), although there appeared to be a tendency toward a higher $K_a$ as well.

Figure 5 demonstrates the relationship between extent of ACC phosphorylation and ACC activity at 0.5 mM citrate. When correlation analysis is performed on data from individual muscles, the value for $R^2$ was 0.78. The hypothesis that the slope of the relationship is 0 was rejected ($P < 0.001$).

Figure 6 shows the relationship between extent of AMPK phosphorylation and the immunoprecipitated AMPK activity. The value for $R^2$ for the individual data points (not means) was found to be 0.59. The probability that the slope of the relationship is 0 was rejected, $P < 0.01$.

Correlation analyses were also performed for the relationship between PCr and ACC activity ($R^2 = 0.72$, $P < 0.001$), phospho-AMPK and phospho-ACC ($R^2 = 0.64$, $P < 0.001$), AMPK activity and ACC activity ($R^2 = 0.39$, $P < 0.001$). The relationships between Cr/PCr and ACC activity and between AMP/ATP and ACC activity appeared to be curvilinear with low correlations ($R^2 = 0.23$ and 0.20, respectively) by use of the linear model.

Effect of treadmill running on phospho-ACC in different types of skeletal muscle. Blood lactate was 1.4 ± 0.2 mM when rats were killed at rest, 2.1 ± 0.1 mM when rats ran at 16 m/min for 10 min, and 3.2 ± 0.3 mM when rats ran at 16 m/min for 5 min followed by 31 m/min for 5 additional minutes. The value in exercising rats was significantly different ($P < 0.05$) from that in resting rats only at the highest work rate. Total AMPK activity (determined on resuspended ammonium sulfate precipitates of muscle) for the red quadriceps was found to be 0.13 ± 0.01, 0.19 ± 0.02, and 0.39 ± 0.04 nmol·g⁻¹·min⁻¹ for resting rats and rats run at 16 m/min and 31 m/min, respectively. For soleus, corresponding values were 0.11 ± 0.01, 0.13 ± 0.01, and 0.18 ± 0.02 nmol·g⁻¹·min⁻¹. Differences...
were significantly different from resting values only at the highest work rate (P < 0.05).

Figure 7 shows the increase in phosphorylation and decrease in ACC activity at 0.5 mM citrate in soleus muscle and in the red region of the quadriceps muscle in rats killed at rest or after running at different speeds on the treadmill for 10 min. Changes were significantly different from rest when rats ran at either 16 or 31 m/min. A high degree of correlation was noted between ACC activity and extent of phosphorylation in both red quadriceps (composed of type IIa fibers) and in soleus (composed predominantly of type I fibers). Neither ACC activity nor ACC phosphorylation was significantly changed in the white region of the quadriceps (data not shown) at these work rates.

DISCUSSION

The principal liver isoform of ACC has been demonstrated to have three sites that are phosphorylated by AMPK: serine 79, serine 1200, and serine 1215 (6, 14, 16, 21). It is phosphorylation of ACC at serine 79 and serine 1200 that has been demonstrated to result in a decline in liver ACC activity (6, 14, 16, 21). The skeletal muscle isoform has not been completely characterized with regard to phosphorylation sites for AMPK, but it is clear that it is a larger protein (2, 15, 45, 49, 56). Although both skeletal muscle and heart isoforms have been grouped together and called ACCβ (or ACC2), it is not entirely certain that heart and skeletal muscle ACC in the rat are identical. PKA has been demonstrated to phosphorylate and inactivate rat heart and liver ACC (10, 16, 21). In liver ACC, serines 77 and 1200 can be phosphorylated by PKA (6, 14, 21). It is clear that muscle ACC can be phosphorylated by PKA, but with no detectable change in ACC activity (55), thus indicating a distinct difference between heart and muscle isoforms. Sequential in vitro phosphorylation studies with PKA and AMPK demonstrate mutual interference, implying the existence of at least

Table 1. Effect of different rates of stimulation on $K_a$ and $V_{\text{max}}$ of gastrocnemius ACC

<table>
<thead>
<tr>
<th>Stimulation Rate</th>
<th>$K_a$, mM</th>
<th>$V_{\text{max}}$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>4.0 ± 0.2</td>
<td>39.0 ± 1.5</td>
</tr>
<tr>
<td>0.2/s</td>
<td>4.7 ± 0.6</td>
<td>33.9 ± 2.2$^*$</td>
</tr>
<tr>
<td>1/s</td>
<td>6.1 ± 0.3$^*$</td>
<td>28.3 ± 1.8$^*$</td>
</tr>
<tr>
<td>5/s</td>
<td>13.8 ± 1.0$^*$</td>
<td>22.8 ± 1.9$^*$</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–10. $^*$P < 0.05.
one common site or of sites in close proximity that physically interfere in muscle ACC (55). Despite the fact that prior phosphorylation with PKA reduces $^{32}$P incorporation from ATP into ACC in response to AMPK treatment, the inactivating effects of phosphorylation by AMPK are not enhanced or prevented. Phosphorylation of the muscle ACC with PKA had no effect on activity, regardless of the order of phosphorylation (55). This would imply that it is a unique AMPK target site (probably equivalent to serine 79 of the liver isoform) that is responsible for activity modulation in muscle ACC and that the site equivalent to serine 1200 in liver ACC is a silent site.

The antibody used to detect phospho-ACC in the present study was prepared against a phospho-peptide sequence surrounding serine 79 of the liver isoform. It is apparent, however, from the present studies, that this antibody also detects phosphorylation of the muscle isoform of ACC. ACC purified from skeletal muscle shows only a faint band in the Western blot for phospho-ACC, indicating that the isolation procedure produces primarily the nonphosphorylated ACC. A very dark band on the Western blot is seen after treatment of the purified ACC with AMPK, AMP, and ATP. No increase in phosphorylation is detected by the phospho-ACC antibody when purified muscle ACC is treated with PKA. Furthermore, in the present studies, a high degree of correlation is noted between ACC activity and phospho-ACC, providing evidence that it is phosphorylation at the site detected by the phospho-ACC antibody that is causing the decline in activity as seen in Figs. 2 and 5.

Previous in vitro studies using $^{32}$P-ATP have demonstrated phosphorylation of purified ACC by AMPK with concurrent decline in extent of citrate activation of ACC, particularly at low citrate concentrations (53). Declines in activity of ACC and changes in the kinetic constants, $K_a$ and $V_{max}$, similar to that induced by phosphorylation in vitro, were observed in muscle of rats running on the treadmill and in gastrocnemius muscles of rats electrically stimulated via the nerve (20, 37, 38, 46, 53). This provided strong evidence that ACC was being phosphorylated by AMPK in contracting muscles. It had not been possible, however, to directly observe phosphorylation of ACC in exercising or stimulated muscle until the specific phospho-ACC antibodies became available.

The first studies utilizing the phospho-ACC antibody were done on muscle biopsies of human subjects. Exercise on the treadmill resulted in marked increases in phosphorylation of ACC (4, 44). Studies in exercising rats and in electrically stimulated rat hindlimb muscles have demonstrated marked decreases in malonyl-CoA corresponding to increases in AMPK activity and decreases in ACC activity (10, 37–39, 46, 50–53). Hindlimb perfusion studies using 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside to activate AMPK also show an inverse relationship between ACC activity (and consequently malonyl-CoA concentration) and fatty acid oxidation (27, 28). Some evidence has been obtained for phosphorylation and activation of malonyl-CoA decarboxylase by AMPK in response to contraction, but purified and recombinant malonyl-CoA decarboxylase does not appear to be a substrate for AMPK in vitro (13, 40).

Studies in human subjects show very low concentrations of malonyl-CoA in resting muscle with little or no change during the course of exercise (7, 32–34). Despite these observations, which tend to discount the functioning of this control system in humans, the fact...
that AMPK is activated and ACC is phosphorylated in exercising human muscle emphasizes the possible importance of this pathway in regulation of fatty acid oxidation. It has been suggested that ACC may be localized near the mitochondrial outer membrane and that local production of malonyl-CoA in the environment of CPT1 may be more important in control of fatty acid oxidation than is the total muscle malonyl-CoA, which does not appear to fluctuate markedly in human muscle during exercise (24, 50).

The present studies on muscle stimulated in situ clearly demonstrate an increase in phosphorylation state of ACC corresponding to a decrease in ACC activity due to activation of AMPK. The measured increase in AMPK activity also corresponds to an increase in phosphorylation of threonine 172 of the α-subunit of AMPK, detected by the phospho-AMPK antibody. In addition to the phosphorylation effect, the AMPK was likely activated allosterically by the increase in free AMP in the muscle and by the decline in the inhibitor, PCr. In fact the allosteric effect may predominate at the lowest stimulation rate (0.2 s⁻¹), because at that frequency a significant decline in ACC activity and increase in phospho-ACC occurred in the absence of a detectable change in AMPK activity or AMPK phosphorylation. A significant decline in muscle content of PCr occurred at this stimulation frequency. Furthermore, the relationship between PCr and ACC activity showed a relatively high correlation coefficient ($R^2 = 0.72$).

The correlation between AMPK activity and ACC activity was relatively low ($R^2 = 0.39$) compared with the correlation between PCr and ACC activity ($R^2 = 0.72$). If ACC activity is considered to be a reporter for the activity of AMPK in the intact muscle, it is reasonable to assume that allosteric activation is responsible for the decline in ACC activity at the lowest stimulation rate. The allosteric activators (AMP) and inhibitors (PCr) would be expected to be discarded during the extensive washing associated with immunoprecipitation of the AMPK. AMP is then added to the reaction mix to maximally activate the enzyme. Only changes in activity due to phosphorylation are detected in this assay.

The absence of a change in ACC activity and phosphorylation in the white quadriceps is not surprising, considering the fact that the low-oxidative type IIB fibers are not likely recruited except at high work rates. Previous studies have demonstrated much smaller changes in glycogen content of the white quadriceps than in the red quadriceps and soleus during the course of treadmill exercise bouts (38, 51).

In summary, the increase in degree of phosphorylation of AMPK at a site detected by an antibody directed against phosphothreonine 172 of the α-subunit is associated with corresponding increasing activity of immunoprecipitated AMPK in contracting gastrocnemius muscle. The increase in degree of phosphorylation of ACC at a site detected by an antibody against serine 79 of liver ACC (the target site for AMPK) correlates well with the decrease in activity of the ACC in three models: purified ACC phosphorylated in vitro by purified AMPK, ACC isolated from gastrocnemius muscles stimulated in situ, and ACC isolated from red quadriceps and soleus muscles of rats running on the treadmill. These studies provide additional information regarding the important role of AMPK in controlling malonyl-CoA concentration and hence fatty acid oxidation in skeletal muscle.

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-41438. Dr. D. G. Hardie provided the purified AMPK.

REFERENCES


