JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Relationship between Kinase Phosphorylation, Muscle Fiber Typing, and Glycogen Accumulation in *Longissimus* Muscle of Beef Cattle with High and Low Intramuscular Fat

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The objective of this study was to examine the association of adenosine monophosphate (AMP)-activated protein kinase (AMPK) with glycogen content in bovine muscle and their links with intramuscular fat (IMF) and muscle fiber type composition. Five steers with high intramuscular fat (High IMF, IMF content is 5.71 ± 0.36%) and five steers with low intramuscular fat (Low IMF, IMF content is 2.09 ± 0.19%) in the *longissimus thoracis* muscle (LM) were selected for immunoblotting, glycogen, and myofiber type composition analyses. The glycogen content was higher in Low IMF muscle than in High IMF muscle (1.07 ± 0.07 versus 0.85 ± 0.08 g/100 g muscle, $P < 0.05$). Phosphorylation of the AMPK α subunit at Thr 172, which is correlated with its activity, was lower ($P < 0.05$) in High IMF compared to Low IMF. In agreement with the lower AMPK phosphorylation in High IMF muscle, the phosphorylation of acetyl–CoA carboxylase (ACC) was also lower ($P < 0.05$) in High IMF muscle than in Low IMF muscle. Glycogen synthase kinase 3 (GSK3) down-regulates glycogen synthesis through phosphorylation of glycogen synthase. The phosphorylation of GSK3 in High IMF was lower ($P < 0.05$) than that in Low IMF, which should down-regulate glycogen synthase activity and reduce the glycogen content in High IMF beef. Type IIB myosin isoform was absent in beef muscle. No noticeable difference in myosin isoform composition was observed between Low and High IMF muscle. In summary, High IMF cattle had lower LM glycogen levels than low IMF cattle, and AMPK activity was less in High IMF than in Low IMF cattle. The difference in glycogen content between Low and High IMF muscle was not correlated with muscle fiber composition. This data shows that LM lipid and glycogen metabolisms are affected by AMPK activity. Thus, AMPK may be a molecular target to alter IMF and glycogen levels in beef muscle.

**KEYWORDS: AMP-activated protein kinase; intramuscular fat; glycogen synthase kinase; glycogen; myosin isoform; beef**

## INTRODUCTION

Marbling is one of the most important factors determining beef quality. Our previous study showed that marbling is correlated with adenosine monophosphate (AMP)-activated protein kinase (AMPK) activity (*1*). AMPK, a heterotrimeric enzyme with α, β, and γ subunits, is mainly recognized as a critical regulator of energy metabolism (*2*, *3*). The α subunit is the catalytic unit, the γ subunit has a regulatory function, and the β unit provides anchorage sites for α and γ (*4*). AMPK is switched on by an increase in the AMP/adenosine 5′-triphosphate (ATP) ratio in muscle cells, which leads to the phosphorylation of AMPK at Thr 172 by AMPKs [including the tumor suppressor gene product LKB-1 (a serine/threonine-protein kinase) and calmodulin-dependent protein kinase kinases] (*2*, *5*, *6*). Activated AMPK inhibits lipogenesis through phosphorylation and inhibition of acetyl–CoA carboxylase (ACC), a key enzyme in lipogenesis. Further, AMPK activity is negatively associated with lipid oxidation (*7*–*9*). Therefore, low AMPK activity is expected to promote lipogenesis and inhibit lipid oxidation, which should increase intramuscular fat (IMF) accumulation.

Excessive antemortem glycogenolysis prior to the slaughter of cattle can result in the formation of beef with a high postmortem muscle pH, commonly referred to as dark-cutting beef (DCB). DCB is characterized by an ultimate muscle pH of 5.87 or greater (*10*), a higher than normal water-holding capacity, and a dark red to almost black lean color (*11*). More importantly, the DCB costs the U.S. beef industry approximately

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10.1021/jf071573z CCC: $37.00 
Published on Web 10/13/2007

$172 million annually or an average loss of $6.08 per steer and heifer slaughtered each year (*12–14*). Reduction in preslaughter stress has been shown to reduce the incidence of DCB (*12, 14–17*). It is plausible that increasing muscle glycogen reserves prior to slaughter may be another method to prevent the formation of DCB; however, up to now, the mechanisms controlling glycogenesis in bovine muscle are poorly defined and understood.

Because AMPK is a master controller of energy metabolism and has been demonstrated to control glycogenolysis in skeletal muscle (*18*) and a high AMPK promotes glucose uptake and glycogen accumulation in muscle (*19, 20*), we hypothesized that AMPK is not only associated with marbling but also with glycogen accumulation in the muscle of beef cattle. Glycogen synthase kinase-3 (GSK3) controls glycogen synthesis through phosphorylation of glycogen synthase. Phosphorylated glycogen synthase is inactive, thereby reducing glycogen synthesis (*21*). Therefore, it is likely that both AMPK and GSK3 are correlated with glycogen content in beef skeletal muscle.

Glycogen content is known to be different in various muscle fiber types. Type II fibers have higher glycogen content compared to type I fibers (*22*). To make things more interesting, the expression of the AMPK$\gamma$3 subunit is muscle fiber type specific, with a high level of expression in type IIb and type IIa fibers and very low level of expression in type I fibers (*23*). Very recently, a fiber specific distribution of AMPK$\alpha$ subunit was also reported (*24*). This prompted us to check the muscle fiber composition of muscle with low and high intramuscular fat (Low and High IMF) content. The objectives of the current study are to determine the correlation of AMPK and GSK-3 with IMF and glycogen accumulation and examine the muscle fiber composition of the bovine skeletal muscle with Low and High IMF content.

## MATERIALS AND METHODS

**Care and Use of Animals.** All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. Calves were vaccinated and branded, and bull calves were castrated according to the University of Wyoming herd processing procedures. Cows and calves grazed the summer pasture at the University of Wyoming McGuire Ranch. Calves were weaned at 155 d of age.

Forty Angus × Gelbvieh steers at 13.5 months of age were transported for one hour to the University of Wyoming Meat Laboratory in the afternoon and slaughtered the next day early in the morning. At the Meat Laboratory, these steers were fasted, with water provided. Immediately after exsanguination and evisceration (less than 10 min postmortem), a sample was removed from the *longissimus thoracis* muscle (LM) between the 12th and 13th rib of the right side of the carcasses by inserting a knife from the body cavity. After trimming off all visible adipose and connective tissues, small pieces of muscle (1 g) were cut and snap frozen in liquid nitrogen for biological analyses. Following IMF analysis, muscle samples from five steers with High IMF (5.71 ± 0.36%) content and five steers with Low IMF (2.09 ± 0.19%) content were selected for further analyses ($P < 0.05$).

**IMF Analyses.** Carcasses were fabricated 7 d postmortem. A 1.3 cm rib steak was removed, vacuum packaged in a high barrier bag, and stored at −29 °C until proximate analysis could be performed. All steaks were trimmed free of fat outside of the epimysium and the bones. Then, the remaining muscles were cut into small pieces and used for IMF analyses by petroleum ether extraction as previously described by Underwood et al. (*1*).

**Antibodies.** Antibodies against AMPK, phospho-AMPK at Thr 172, ACC, phospho-ACC at Ser 79, GSK-3, and phospho-GSK3 at Ser 21/9 were purchased from Cell Signaling (Danvers, MA). Anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was bought from ABcam Inc. (Cambridge, MA).

**Immunoblot Analysis.** Muscle sample (0.1 g) was powdered in liquid nitrogen and homogenized in a polytron homogenizer (7 mm diameter generator) with 400 $\mu$L of ice-cold buffer containing 137 mM NaCl, 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), 2% sodium dodecyl sulfate (SDS), 1% NP-40, 10% glycerol, 2 mM PMSF, 10 mM sodium pyrophosphate, 10 $\mu$g/mL aprotinin, 10 $\mu$g/mL leupeptin, 2 mM $Na_3VO_4$, and 100 mM NaF (pH 7.4). The protein content of lysates was determined by the Bradford method (Bio-rad Laboratories, Hercules, CA) (*25*).

Muscle homogenate was mixed with an equal volume of 2 × standard SDS sample loading buffer. A Bio-rad mini-gel system was used for casting gels and running electrophoresis. Five to 20% gradient gels were used for SDS-polyacrylamide gel electrophoresis (PAGE) separation of proteins. After electrophoresis, the proteins on the gels were transferred to nitrocellulose membranes in a transfer buffer containing 20 mM tris(hydroxymethyl)aminomethane (Tris)-base, 192 mM glycine, 0.1% SDS, and 20% methanol. Membranes were incubated in a blocking solution consisting of 5% nonfat dry milk in TBS/T (0.1% Tween-20, 50 mM Tris-HCl pH 7.6, and 150 mM NaCl) for 1 h. Membranes were incubated overnight in primary antibodies with 1:1000 dilution in TBS/T with 2% bovine serum albumin (SeraCare Diagnostics, Milford, MA). After the primary antibody incubation, membranes were washed three times for 5 min each with 20 mL of TBS/T. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilution for 1 h in TBS/T with gentle agitation. After three 10 min washes, membranes were visualized using ECL Western blotting reagents (Amersham Bioscience) and exposed to film (MR, Kodak, Rochester, NY). The density of the bands was quantified by using an Imager Scanner II and the ImageQuant TL software (*26*). To reduce the variation between blots, cell lysates of all treatments were run in a single gel. The band density among different blots was normalized according to the density of the reference band. The band density was also normalized according to the GAPDH content.

**Glycogen Content Measurement.** Glycogen was assayed using a muscle sample (0.1 g) powdered in liquid nitrogen. Glycogen, glucose, and glucose 6-phosphate were determined as previously reported by Shen et al. (*27*) Glycogen content was calculated using the equations of Keppler and Decker (*28*). Glycogen is expressed as g of glycogen per 100 g of wet LM tissue.

**Muscle Fiber Typing.** Muscle fiber typing was conducted as described previously using a method that distinguishes between fiber types on the basis of myosin ATPase activity under various preincubation pH conditions (*29*). Briefly, serial cryostat sections (8 $\mu$m) of the LM were incubated in either a pH 4.2 (0.2 M barbital acetate) solution and pH 4.5 (0.2 M barbital acetate) solution for 5 min or a pH 10.5 (33 mM sodium barbital, 60 mM $CaCl_2$) solution for 15 min. Sections were then rinsed once with water, soaked in ATP solution (25 min for the pH 4.2 and 4.5 solutions and 15 min for the pH 9.7 solution), washed three times with 1% $CaCl_2$, and then incubated with 2% cobalt chloride for 10 min. After that, sections were washed with diluted 0.1 M sodium barbital and then with water. Finally, sections were soaked in 2% (v/v) ammonium sulfide for 20–30 s and washed with water. The sections were observed using light microscopy (*29*).

**Identification of Myofiber Isoforms.** *Longissimus* muscle samples (0.1 g) were homogenized in 500 $\mu$L of buffer containing 250 mM sucrose, 100 mM KCl, 5 mM ethylenediaminetetracetic acid (EDTA), and 20 mM Tris-HCl pH 6.8. The homogenate was filtered through nylon cloth to remove debris and centrifuged at 10 000 × *g* for 10 min. The pellet obtained was re-suspended in 500 $\mu$L of washing buffer containing 200 mM KCl, 5 mM EDTA, 0.5% Triton X-100, and 20 mM Tris-HCl pH 6.8. The suspension was centrifuged at 10 000 × *g* for 10 min. The pellet containing purified myofibrillar proteins was re-suspended in 200 $\mu$L of water and 300 $\mu$L of standard 2 × sample loading buffer and then boiled for 5 min. After centrifugation at 12 000 × *g* for 5 min, the supernatant was used for electrophoresis.

The stacking gels consisted of 4% acrylamide (acrylamide/bis = 50:1) and 5% (v/v) glycerol, 70 mM Tris-HCl (pH 6.7), 0.4% (w/v) SDS, 4 mM EDTA, 0.1% (w/v) APS, and 0.01% (v/v) TEMED. The separation gel contained 5% (w/v) glycerol, acrylamide/bis (50:1) at a concentration ranging from 5 to 20%, 200 mM Tris pH 8.8, 4 mM EDTA, 0.4% (w/v) SDS, 0.01% (v/v) TEMED, and 0.1% (w/v) ammonium persulfate. The upper running buffer consisted of 0.1 M

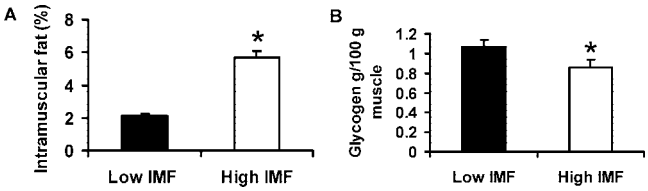


**Figure 1.** IMF and glycogen content in *longissimus thoracis* muscle of Low IMF (■) and High IMF (□) cattle. Panel **A**: IMF content. Panel **B**: glycogen content. (*) $P < 0.05$. Mean ± SE ($n = 5$ per group).

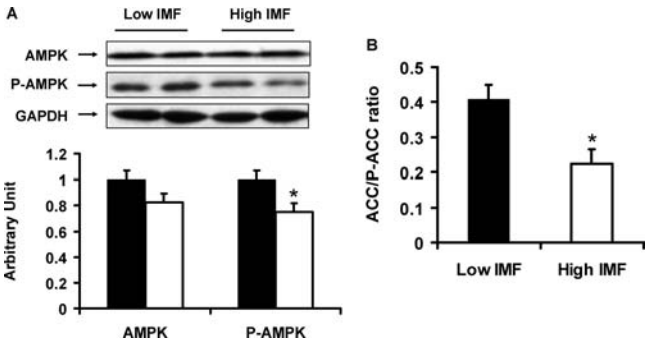


**Figure 2.** AMPK, the ratio of phosphorylated ACC, and the total ACC in *longissimus thoracis* muscle of Low IMF (■) and High IMF (□) cattle. Panel **A**: AMPK and phospho–AMPK immunoblots and statistical data of band density. Panel **B**: ratio of phosphorylated ACC to total ACC. (*) $P < 0.05$. Mean ± SE ($n = 5$ per group).

Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 150 mM glycine, and 10 mM mercaptoethanol; the lower running buffer consisted of 50 mM Tris-HCl (pH 8.8), 0.01% (w/v) SDS, and 75 mM glycine. Gels were run at 8 °C in a Hoefer SE 600 (Hoefer Scientific, San Francisco, CA) unit, at constant 200 V for 24 h (*30*). After electrophoresis, gels were stained with Coomassie blue and scanned with a densitometer to determine the amount of each myosin isoform, and the percentage composition was reported. In addition, the identification of myosin isoforms was confirmed by immunoblotting analyses using an antibody against myosin heavy chain (DSHB, Iowa City).

**pH Analysis.** Muscle samples (0.1 g) were homogenized in 1000 μL of deionized water, and homogenates were immediately used for pH measurement using a Beckman pH meter (Model PHI71, Beckman Instruments, Inc., Fullerton, CA) equipped with a sympHomv probe (VWR Scientific, West Chester, PA).

**Statistical Analysis.** Data were analyzed as a completely randomized design using the PROC GLM (General Linear Model of Statistical Analysis System, SAS, 2000). An individual animal was considered as an experimental unit. The differences in the mean values were compared by Tukey's multiple comparison method, and mean ± standard errors were reported. Statistical significance was considered as $P < 0.05$.

## RESULTS AND DISCUSSION

**AMPK, Marbling, and Glycogen Accumulation.** On the basis of human and rodent studies, AMPK and GSK-3 are two kinases controlling glycogen accumulation in skeletal muscle. Activation of AMPK switches on pathways that generate ATP, such as glycolysis and fatty acid oxidation, while switching off ATP-consuming processes such as the synthesis of glycogen and fatty acids (*31*). High AMPK activity promotes glucose uptake and thus increases glycogen accumulation in muscle (*20, 32*); meanwhile, AMPK promotes fatty acid oxidation. Because fatty acid oxidation and synthesis are expected to affect IMF content, this prompted us to hypothesize that muscle glycogen content and marbling are negatively correlated and that AMPK is involved in both processes. Data obtained in this study support our hypothesis. The average IMF content for Low IMF was 2.09 ± 0.19% and for High IMF was 5.71 ± 0.36% ($P < 0.01$, **Figure 1A**). The glycogen content was also higher in Low IMF muscle than in High IMF muscle (1.07 ± 0.07 versus 0.85 ± 0.08 g/100 g muscle, **Figure 1B**). The pH values of Low IMF (5.35 ± 0.70) and High IMF (5.34 ± 0.09) muscle were similar ($P = 0.42$). Additionally, there were no carcasses with ultimate pH ≥ 5.87, a hallmark for DCB (*10*). Further information regarding the fat thickness, carcass weight, grades, and muscle area of High and Low IMF beef cattle were reported elsewhere (*1*).

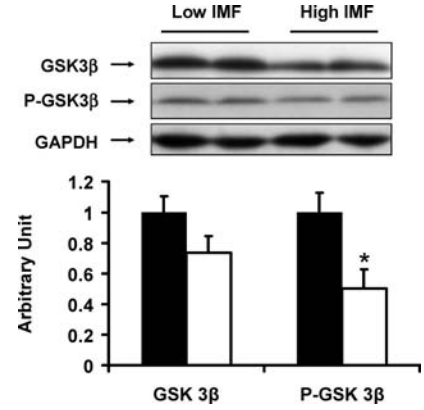


**Figure 3.** Glycogen synthase kinase-3$\beta$ (GSK3$\beta$) and its phosphorylation at Ser 21/9 (P-GSK3$\beta$) in *longissimus thoracis* muscle of Low IMF (■) and High IMF (□) cattle. (*) $P < 0.05$. Mean ± SE ($n = 5$ per group).

To explore associated mechanisms, we analyzed AMPK activity in these muscle samples. We used AMPK phosphorylation at Thr 172 and ACC phosphorylation at Ser 79 to assess AMPK activity, which have been used in previous publications (*33–37*). In our previous study, the phosphorylation of AMPK and ACC was highly correlated with AMPK activity (*38*). Thus, in this study, AMPK and ACC phosphorylation was used to substitute AMPK activity analyses. As expected, the phosphorylation of AMPK was 18.8 ± 5.4% lower in High IMF than in Low IMF ($P < 0.05$, **Figure 2A**). In agreement with the lower AMPK phosphorylation in High IMF muscle, the ratio of ACC phosphorylation was also 45.5 ± 10.0% lower in High IMF muscle than in Low IMF muscle ($P < 0.05$, **Figure 2B**). These data clearly showed that AMPK activity was decreased in High IMF muscle compared to Low IMF muscle.

AMPK is a crucial kinase controlling glucose metabolism (*3, 4*). AMPK regulates glycogen synthesis and utilization through phosphorylation of phosphorylase kinase. This phosphorylation leads to its activation, which further activates glycogen phosphorylase by phosphorylation (leading to glycogenolysis) (*39–41*). However, in the other end, a high AMPK activity promotes glucose uptake that enhances glycogen accumulation (*20, 32*). Therefore, the low AMPK activity in High IMF muscle should reduce the accumulation of glycogen.

Acetyl–CoA carboxylase is an enzyme which catalyzes the synthesis of malonyl–CoA, a key substrate for lipogenesis. The phosphorylation of ACC by AMPK inhibits ACC activity. Hence, the reduced ACC phosphorylation in High IMF muscle promotes lipogenesis, which may provide an explanation for the High IMF content in these cattle. These data show an important role of AMPK in both glycogen utilization and fat accumulation, suggesting that AMPK might be a target for improving beef quality.

**GSK3 and Glycogen Accumulation.** GSK-3 is a serine/threonine kinase that negatively regulates glycogen synthase activity by phosphorylation. Because GSK-3 is constitutively active (not phosphorylated) in unstimulated muscle cells,

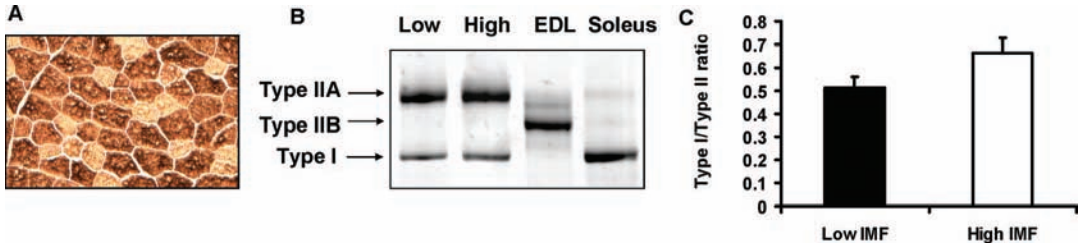


**Figure 4.** Myosin isoforms and muscle fiber typing of LM of Low IMF (■) and High IMF (□) cattle. Panel **A**: muscle fiber typing by preincubation at pH 10.5. Panel **B**: myosin isoform separation by electrophoresis. Soleus: *soleus* muscle from rats. EDL: *extensor digitorium longus* muscle from rats. Panel **C**: ratio of type I to type II myosin fibers. Mean ± SE ($n = 5$ per group).

glycogen synthase primarily exists in a phosphorylated and inactive state (*21*). However, in response to the stimulation of insulin, GSK-3 is itself phosphorylated and inactivated by protein kinase B (Akt), a main kinase activated by insulin receptor mediated signaling (*21, 42*). Because of a lack of phosphorylation catalyzed by GSK3, glycogen synthase is gradually dephosphorylated by protein phosphatases and becomes active (*21*). The phosphorylation of GSK-3 in High IMF was 49.8 ± 12.6% lower than that in Low IMF ($P < 0.05$, **Figure 3**), which should inhibit glycogen synthase activity and reduce glycogen accumulation in High IMF consistent with the lower glycogen content in High IMF compared to that in Low IMF.

**Myosin Isoform Composition and Muscle Fiber Typing.** We used a standard myosin ATPase staining method to identify the fiber type composition. Following preincubation at pH 10.5, type I muscle fibers were only weakly stained but type II muscle fibers were stained strongly resulting in a dark appearance (**Figure 4A**). However, we could not clearly differentiate type IIA and IIB muscle fibers despite our numerous trials using different preincubation pH values. This is in agreement with previous reports that the myosin ATPase staining technique only allowed the identification of type I and II muscle fibers (*43–45*) in sheep. In addition, it was shown using immunoblotting that the type IIb myosin isoform is absent in sheep muscle (*46*). We examined the myosin isoform composition in beef muscle samples. As shown in **Figure 4B**, there were clear bands of type I and type IIA isoforms, but no type IIB myosin isoform was detected. Rat *extensor digitorium longus* (EDL) muscle is mainly composed of type IIB muscle fibers, and *soleus* muscle is mainly made of type I muscle fibers (*47*). To confirm our identification of isoforms, rat EDL and *soleus* muscle were loaded on the same gel with beef muscle samples. As expected, EDL muscle showed a strong type IIB band, and *soleus* muscle showed a strong type I band (**Figure 4B**). However, the same region is blank for bovine LM, indicating that cattle LM lacks the expression of type IIB myosin isoform. To eliminate any chance of misidentification of other bands as myosin bands, we used immunoblotting with an antibody against myosin heavy chain, which confirmed that those bands are myosin isoform bands (results not shown). To the authors knowledge, this is the first report demonstrating that bovine muscle lacks the expression of type IIB myosin isoform. We realize that this is a small sample size only representing british × continental crossbreds, so this cannot be assumed for all breeds of cattle with an absence of type IIB myosin isoform. We further quantified the ratio of type I to type II muscle fibers in cattle muscle. There was no significant difference between Low IMF and High IMF muscle ($P = 0.09$, **Figure 4C**). These data showed that fiber type composition might not be the main reason for the different glycogen and fat accumulation in Low and High IMF muscle of beef cattle.

In summary, our data demonstrate that High IMF beef muscle had lower glycogen levels than Low IMF beef muscle and that AMPK activity was lower in High IMF versus Low IMF muscle. The GSK3 activity was lower in High IMF muscle than in Low IMF muscle. No type IIB myosin isoform was detected in the beef muscle of these british × continental steers, and in spite of the differences in glycogen levels, the ratio of type I to type II fibers did not differ between High and Low IMF cattle muscle.

**Implications.** AMPK may provide a molecular target to increase the IMF of cattle as it was negatively associated with IMF. Additionally, the amount of glycogen in muscle may be manipulated by changing AMPK activity. Recently, the identification of several plant compounds activating AMPK (*48, 49*) provides promise that marbling and glycogen content in beef cattle may be managed through dietary supplementation. However, additional studies are needed to develop strategies for manipulation of AMPK activity in vivo.

## ACKNOWLEDGMENT

The authors thank Jessica M. Kimzey for her assistance in myosin fiber typing.

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**Received for review May 29, 2007. Revised manuscript received August 13, 2007. Accepted August 18, 2007. This work was supported by Research Initiative Grants 2007-35203-18065, 2006-55618-16914, 2004-35503-14792, and 2003-35206-12814 from the USDA Cooperative State Research, Education and Extension Service. The monoclonal antibody of actin developed by Jim Jung-Ching Lin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.**

JF071573Z