ROLE OF AMPK SUBUNITS IN SKELETAL MUSCLE mTOR SIGNALING

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ABSTRACT
AMP-activated protein kinase (AMPK) is an important energy sensing protein in skeletal muscle. Mammalian target of rapamycin (mTOR) mediates translation initiation and protein synthesis through ribosomal S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1). AMPK activation reduces muscle protein synthesis by downregulating mTOR signaling, while insulin mediates mTOR signaling via Akt activation. We hypothesized that AMPK-mediated inhibitory effects on mTOR signaling depend on catalytic α2 and regulatory γ3 subunits. Extensor digitorum longus (EDL) muscle from AMPK α2 knockout (KO), AMPK γ3 KO, and respective wild-type (WT) littermates (C57Bl/6) were incubated in the presence of 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), insulin, or AICAR plus insulin. Phosphorylation of AMPK, Akt and mTOR-associated signaling proteins were assessed. Insulin increased Akt Ser473 phosphorylation (p<0.01), irrespective of genotype or presence of AICAR. AICAR increased phosphorylation of AMPK Thr172 (p<0.01) in WT, but not KO mice. Insulin-stimulation increased phosphorylation of S6K1 (Thr389), rpS6 (Ser235/236) and 4E-BP1 (Thr37/46) (p<0.01) in WT, AMPK α2 KO and AMPK γ3 KO mice. However, in WT mice, pre-incubation with AICAR completely inhibited insulin-induced phosphorylation of mTOR targets, suggesting mTOR signaling is blocked by prior AMPK activation. The AICAR-induced inhibition was partly rescued in EDL muscle from either α2 or γ3 AMPK KO mice, indicating functional α2 and γ3 subunits of AMPK are required for the reduction in mTOR signaling. AICAR alone was without effect on basal phosphorylation of S6K1 (Thr389), rpS6 (Ser235/236) and 4E-BP1. In conclusion, functional α2 and γ3 AMPK subunits are required for AICAR-induced inhibitory effects on mTOR signaling.

INTRODUCTION
The balance between nutrient overload and shortage is a constant challenge to energy homeostasis in living organisms. In mammalian cells, energy homeostasis is tightly regulated by growth factors, hormones, and nutrients that evoke evolutionary conserved signaling pathways (1). The 5’AMP-activated protein kinase’- (AMPK) is a ubiquitously expressed serine/threonine protein kinase that is a master regulator of energy homeostasis (2, 3). AMPK is activated during states of energy stress such as hypoxia, glucose starvation or physical exercise, and restores the energy depleted status by concomitantly inhibiting anabolic, and stimulating catabolic pathways (3-5). Protein synthesis, a major consumer of ATP in mammalian cells, is inhibited upon AMPK activation (1).

The mammalian target of rapamycin (mTOR) signaling pathway plays a key role in the regulation of protein synthesis (6). Insulin stimulates protein synthesis via activation of the canonical PI 3-kinase/Akt pathway. Akt phosphorylates and inactivates the tuberous sclerosis complex (TSC2), which increases mTOR kinase activity. mTOR exist in two protein complexes; mTORC1 consist of mTOR, the G-protein beta-like protein (GβL/LST8) and raptor, and is responsible for cell growth, whereas mTORC2 contains mTOR, GβL and rictor, and is important in cytoskeletal organization. Ribosomal S6 kinase 1 (S6K1) and eIF4E binding protein 1 (4E-BP1) are well-characterized substrates of mTOR. The phosphorylation state of these substrates reflects the activity of the mTOR-raptor branch of the pathway. Phosphorylation of 4E-BP1 inhibits binding of 4E-BP1 with mRNA cap binding protein, eIF4E, thereby allowing the association of eIF4E with eIF4G to initiate translation (6-8). Upon activation, S6K1 phosphorylates ribosomal protein S6 (rpS6), a component of the 40S ribosomal subunit complex, and increases ribosomal biogenesis through translation of a subclass of mRNAs containing a short oligopyrimidine sequence (9), although this mechanism has been challenged (10).

Skeletal muscle mTOR signaling is upregulated in response to resistance exercise, growth factor stimulation, and high-protein diet, which promotes adaptive changes in skeletal muscle mass that correlate with increased mTOR activity (11, 12). AMPK is a negative regulator of mTOR signaling, and therefore may play a role in the regulation of skeletal muscle hypertrophy. AMPK is a heterotrimeric complex, consisting of the catalytic α subunit and the regulatory β and γ subunits. AMPK α and β subunits are each encoded by two distinctive genes (α1, α2 and β1, β2), whereas the γ subunit is encoded by three genes (γ1, γ2 and γ3). Twelve different combinations of AMPK subunits form haloenzymes with tissue-specific distribution and subcellular localization.
Binding of AMP to γ subunits allosterically activates AMPK, which promotes phosphorylation of threonine residue (Thr172) within the activation domain of the α subunit by the upstream tumor suppressor, LKB1 kinase (2, 4, 13, 14). The adenosine analogue 5-aminimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), which is metabolized to ZMP, can mimic the effect of AMP to activate AMPK (15).

The existence of 12 possible AMPK heterotrimers complicates the understanding of the physiological role of the AMPK system. Tissue-specific relative expression patterns of AMPK subunits in different species may explain the relative contribution of different subunits of AMPK to specific metabolic responses (16-20). The predominant AMPK heterotrimeric complex expressed in glycolytic skeletal muscle contains the α2/β2/γ3 subunits (20). Functional α2 and γ3 AMPK subunits are required for AICAR-induced glucose transport in glycolytic skeletal muscle (21, 22). While AMPK subunit-specific effects on mTOR signaling have been reported for different cell lines (23, 24), effects in skeletal muscle are unclear. We determined the role of AMPK in mTOR signaling using knock-out mouse models in which either the α2 (25) or the γ3 (21) subunit has been ablated. We examined the effect of insulin on downstream targets of mTOR and the inhibitory effect of AMPK on insulin-induced mTOR signaling. We also determined whether AICAR-induced inhibition of insulin-mediated mTOR signaling is intact in α2-AMPK knockout (KO) and γ3-AMPK KO mice. Our results suggest that functional α2 and γ3 subunits of AMPK are required for the AMPK-mediated inhibitory effect on insulin-induced mTOR signaling.

**RESULTS**

**AICAR-induced AMPK phosphorylation requires functional α2 and γ3 subunits-** Insulin (120 nM) and AICAR (2 mM) were used as pharmacological activators (6) and repressors (26) of mTOR signaling, respectively. To study the inhibitory effect of AMPK on mTOR signaling, EDL muscles from α2 AMPK KO, γ3 AMPK KO, and their corresponding wild-type littermates (WT) (C57Bl/6), were incubated in the presence or absence of AICAR (60 min). Immunoblot analysis with a phospho-AMPK Thr172 antibody revealed that AICAR increased AMPK phosphorylation in EDL muscles from WT animals and this effect was unaltered by insulin. However AICAR-induced phosphorylation of AMPK was completely blunted in EDL muscle from α2 AMPK KO and γ3 AMPK KO mice (Figure 1, 2A and 2B). Thus, AICAR-induced AMPK phosphorylation requires functional α2 and γ3 AMPK subunits. Irrespective of genotype, insulin alone or in combination with AICAR was without effect on AMPK phosphorylation, consistent with our previous studies (21, 27).

Akt signaling is unaltered in α2 AMPK KO and γ3 AMPK KO mice- EDL muscle from α2 AMPK KO, γ3 AMPK KO and WT mice were incubated in the absence or presence of 120 mM insulin (30 min). Insulin increased Akt Ser473 phosphorylation independent of either genotype or exposure to AICAR. In all mouse models, AICAR was without effect on Akt Ser473 phosphorylation (Figure 1, 2C and 2D). These results support our earlier findings (27) that insulin signaling to Akt does not require the α2 or γ3 AMPK subunit.

**AICAR-induced inhibition of mTOR signaling requires functional AMPK α2 and γ3 subunits-** Insulin stimulation induced a profound increase in phosphorylation of S6K1 Thr389, rpS6 Ser235/236 and 4E-BP1 Thr37/46 in WT, α2 AMPK KO and γ3 AMPK KO. However, in EDL muscle from WT mice, pre-incubation with AICAR completely inhibited insulin-induced phosphorylation of all mTOR targets, suggesting that mTOR signaling is blocked by AMPK activation. This inhibition was partly rescued in EDL muscle from α2 AMPK KO and γ3 AMPK KO mice, indicating that functional α2 and γ3 subunits of AMPK are required for the AMPK-mediated inhibition of mTOR signaling. AICAR alone was without effect on the basal phosphorylation of S6K1 Thr389, rpS6 Ser235/236 or 4E-BP1 (Figure 1, 3A-F). Similar patterns of phosphorylation were also noted for 4E-BP1 Thr37/46, although this did not achieve statistical significance. Expression of S6K1, rpS6 and 4E-BP1 was similar between genotypes and unaltered by the in vitro treatments (data not shown).

**DISCUSSION**

AMPK suppresses mTOR signaling and protein synthesis (24, 26, 28), via phosphorylation and activation of TSC2 (Thr1457 and Ser1345) (29)
and/or phosphorylation and inactivation of mTOR on Thr\textsuperscript{2446} (30). Using two animal models in which the predominant catalytic and regulatory AMPK isoforms expressed in glycolytic skeletal muscle have been genetically manipulated, we provide direct evidence linking AMPK subunits to mTOR signaling. AICAR-induced activation of AMPK in EDL muscle of α2 AMPK KO and γ3 AMPK KO mice was markedly impaired, indicating α2 and γ3 subunits are critical for the activation of AMPK.

Following insulin/growth factor-mediated activation; Akt stimulates mTOR signaling via phosphorylation and inactivation of TSC2 (Thr\textsuperscript{389}) (31) and/or phosphorylation of mTOR (Ser\textsuperscript{2448}) (30). Insulin signaling was unaltered in α2 AMPK KO and γ3 AMPK KO mice, and AICAR was without effect on phosphorylation of Akt in the absence of insulin. Our results provided evidence against cross-talk between AMPK and Akt signaling pathways under basal or insulin-stimulated conditions.

To determine whether AMPK and mTOR signaling pathways intersect, skeletal muscle was exposed to a combination of insulin and the AMPK activator, AICAR and S6K1 and 4E-BP1 were studied. Phosphorylation of eEF2K and its substrate eEF2 (eukaryotic elongation factors) were unaltered in skeletal muscle (data not shown). In contrast, AICAR repressed insulin-induced phosphorylation of mTOR (Ser\textsuperscript{2448}) in wild-type mice, while this effect was partially blocked in α2 AMPK KO and γ3 AMPK KO mice (Figure 1).

mTOR regulates protein synthesis by two common mechanisms; first it activates S6K1, which is a positive regulator of protein translation and second, it phosphorylates and inhibits 4E-BP1 activity. A negative regulator of the protein initiation factor eIF-4E (7, 8). Activity of S6K1 is regulated by multiple site phosphorylation. Thr\textsuperscript{389} phosphorylation is rapamycin-sensitive via the mTOR/raptor complex and appears to be a critical rate-limiting step for mTOR activation (25, 32). The rpS6 protein is the first identified substrate of S6K1, and its phosphorylation is directly associated with activation of S6K, as well as regulation of cell size (33). Insulin phosphorylates S6K1 Thr\textsuperscript{389} and rpS6 Ser\textsuperscript{235/236} in wild-type, α2 AMPK KO and γ3 AMPK KO mice, providing evidence that S6K1 is activated and correlated with Akt phosphorylation (Figure 3A-D and 2C-D). Therefore, activation of the Akt-S6K1 pathway is independent of AMPK α2 and γ3 subunits in skeletal muscle. However AICAR blocked insulin-mediated phosphorylation of S6K1 and rpS6 in wild-type mice (Figure 1 and 3), suggesting opposing effects of Akt and AMPK on S6K1 activity. In α2 AMPK KO and γ3 AMPK KO mice, the inhibitory effect of AICAR was blocked; clearly indicating AMPK-mediated inhibitory signals are integrated through α2 and γ3 subunits. Interestingly, activity of the mTOR pathway is increased in obese insulin resistant rodents, whereas depletion of S6K1 protects against diet-induced insulin resistance (34). S6K deficient skeletal muscle is associated with an activation of AMPK, and AMPK inhibition restores cell growth and sensitivity to nutrient signals (35).

Our results support the hypothesis that cross-talk at the level of AMPK and mTOR are critical for cellular energy homeostasis in health and disease.

4E-BP1 is subjected to series of hierarchical phosphorylation events induced by mTOR (7, 8). Phosphorylation of 4E-BP1 Thr\textsuperscript{374/46} as it is an initial and important step in mTOR signaling. Insulin phosphorylates 4E-BP1 in wild-type, α2 AMPK KO and γ3 AMPK KO mice, with effects positively correlated with the degree of Akt phosphorylation (Figure 3E-F). AICAR preincubation completely blocked the insulin-mediated phosphorylation of 4E-BP1 in wild-type, α2 AMPK KO and γ3 AMPK KO mice, which tightly correlates with AMPK phosphorylation. Though 4E-BP1 phosphorylation was not significantly increased, in both knockout models the phosphorylation pattern was strikingly similar to that observed for S6K1 and rpS6 phosphorylation. The role of 4E-BP1 in protein synthesis remains obscure, mainly because disruption of the 4E-BP1 gene does not alter growth and protein synthesis (36).

Our results suggest that AMPK α2 and γ3 are partly required for the inhibitory effects on insulin-stimulated phosphorylation of 4E-BP1.

The inhibitory effect of AMPK on insulin-induced mTOR signaling is physiological relevant, since the γ3 isoform is mainly found in complexes containing α2 and β2 subunits in mouse glycolytic skeletal muscle (20). However the inhibitory effects of AMPK on insulin-induced mTOR signaling are incompletely prevented in α2 AMPK KO and γ3 AMPK KO mice (Figure 3). The expression of other AMPK subunits is unaltered γ3 AMPK KO mice (21).
Conversely, expression of β1, β2 and γ3 is decreased and α1 is increased in α2 AMPK KO mice (37). However, the increase in α1 subunit expression in α2 AMPK KO mice does not restore total AMPK activity in α2 AMPK KO mice (37). Nevertheless, changes in AMPK subunit expression may possibly explain why the inhibitory effects of AICAR on insulin-induced mTOR signaling are almost completely prevented in α2 AMPK KO mice compared to γ3 AMPK KO mice.

Glycolytic skeletal muscle such as the EDL expresses α1 and γ1 subunits of AMPK (20), which might contribute to the AICAR-induced inhibition of mTOR signaling in α2 AMPK KO and γ3 AMPK KO mice. Compounded KO mice, whereby α2 and γ3 subunits are deleted or β2 AMPK KO mice may show a prominent rescue effect of AICAR on insulin-induced mTOR signaling. While the major support for repressive effects of AMPK on mTOR signaling comes from experimental approaches where AICAR has been used as pharmacological activator of AMPK, we cannot exclude the possibility that AICAR may act independently of AMPK activation through intracellular P(i) depletion and ZMP accumulation (38).

Skeletal muscle hypertrophy is associated with resistance exercise, increased mechanical load, Akt activation, mTOR signaling and increase rates of protein synthesis, whereas nutrient overload induces hypertrophy in Akt-independent manner (12). During endurance exercise the AMP:ATP ratio progressively increases, which leads to activation of AMPK (39). Though increased AMPK activity is associated with decreased muscle hypertrophy (28), skeletal muscle specific knockout of LKB1, the upstream activator for AMPK, is associated with an increase in cell size (40). Activation of TSC2 by AMPK is dominant over PKB-mediated inactivation and leads to the inactivation of mTOR and a decrease in protein synthesis (29). The temporal relationship between Akt and AMPK might be central to the regulation of mTOR signaling and skeletal muscle mass.

The regulation of mTOR is complex because it is a point of convergence, which integrates several signals (7, 8, 41, 42). Defect in mTOR signaling is associated with several diseases including diabetes and cancer. Our results provide evidence that AMPK is negative regulator of mTOR signaling in skeletal muscle. We reveal AMPK complexes containing the α2 and γ3 subunits are required for the inhibitory effect of AMPK on mTOR signaling in glycolytic muscle. Thus, AMPK is an important regulator of mTOR signaling, that provides a level of cross-talk and feedback inhibition on downstream signaling towards mTOR.

**MATERIALS AND METHODS**

*Animals*- All experiments were approved by the Regional Animal Ethical Committee (Stockholm, Sweden) and the Danish Animal Experimental Inspectorate. Four to five month-old female mice from three different strains were used: α2-AMPK knockout (KO) (25), γ3-AMPK KO (21), and C57Bl/6 mice. WT littermates were used as controls for each genetic model studied. Within each knockout strain, mice of different genotypes were produced by intercross-breeding (hetero-hetero for α2 and γ3). Mice were maintained on a 10 h/14 h light/dark cycle and received standard rodent chow.

*Muscle incubation procedures*- Mice were anesthetized via intraperitoneal injection of 2.5% avertin (0.02 ml/g of body weight), or pentobarbital (6 mg/100 g body weight), and extensor digitorum longus (EDL) muscles were quickly removed and incubated in Krebs-Henseleit pre-buffer at 30°C oxygenated with a gas containing 95% O2 and 5% CO2. The content of the pre-buffer has been described previously (21, 25). Incubations were carried out using pre-buffer, to which either AICAR (2 mM; Toronto Research Chemicals inc., Toronto, Canada), or insulin (120 nM; Actrapid, Novo Nordisk, Bagsværd, Denmark) was added. All muscles were pre-incubated for 30 min in pre-buffer followed by four different incubation conditions - Basal (control), insulin, AICAR or AICAR plus Insulin. The total incubation time for AICAR and insulin was 60 min and 30 min respectively. In order to check the inhibitory effect of AMPK on insulin-mTOR signaling, muscles were preincubated with AICAR (30 min) and subsequently by with AICAR plus insulin (30 min). After incubation, muscles were harvested, washed in ice-cold Krebs-Henseleit buffer, blotted on filter paper, and quickly frozen with aluminum tongs pre-cooled in liquid nitrogen and stored at -80°C.

*Muscle lysate preparation*- Muscles were homogenized in ice-cold buffer (10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl,
50 mM Hepes (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 2 mM sodium orthovanadate, 3 mM benzamidine, pH 7.4) for 20 sec using a motor-driven pestle. Homogenates were rotated end-over-end for 1 h at 4˚C and subjected to centrifugation (14000 g for 10 min) at 4˚C. The supernatant was frozen in liquid nitrogen and stored at -80˚C. Protein content in lysates was measured by the bicinchoninic acid method (Pierce, Rockford, IL).

Immunoblot analysis- Phosphorylation and expression of various proteins were determined by using following antibodies against phospho AMPK Thr\(^{172}\), phospho Akt Ser\(^{473}\), phospho p70S6K Thr\(^{389}\), phospho rpS6 Ser\(^{235/236}\), phospho 4E-BP1 Thr\(^{37/46}\) (Cell Signaling Technology). A pan-actin (Cell Signaling Technology) antibody was used to confirm equal loading. Muscle lysates were adjusted to equal protein concentration and boiled in Laemmli-buffer and loaded on 7.5%, 12% or 6-12% gradient gels and transferred to a polyvinylidenedifluoride membrane (Immobilon Transfer Membrane, Millipore). Membranes were blocked in TBST buffer (10 mM Tris-base, 150 mM NaCl, 0.25% Tween 20) containing 5% low fat milk protein for 2 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4˚C, washed with TBST buffer followed by incubation with appropriate horse-radish peroxidase-conjugated secondary antibody (Bio-Rad, Richmond, CA) for 1h at room temperature. Immuno-reactive proteins were visualized by enhanced chemiluminescence (ECL or ECL plus; Amersham, Arlington Heights, IL) and quantified by densitometry using Molecular Analyst Software (Bio-Rad) and results were expressed as relative units compared with basal samples loaded on each gel.

Statistics- Data are expressed as mean ± SEM. Statistical evaluation was performed by Two-Way ANOVA and Tukey’s post hoc analysis were applied to identify significant differences between groups when effects were statistical significant. All statistical analyses were performed using SigmaStat 3.5 (Systat Software Inc., CA). p<0.05 was considered significant.

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FIGURE LEGENDS

Figure 1. Representative immunoblot showing effect of insulin, AICAR or AICAR plus insulin on phosphorylation of AMPK Thr\(^{172}\), pAkt Ser\(^{473}\), p-mTOR Ser\(^{2448}\), pS6K1 Thr\(^{389}\), p-rpS6 Ser\(^{235/236}\) and p4E-BP1 Thr\(^{37/46}\) in EDL muscle from C57Bl/6 (WT), α2 AMPK KO and γ3 AMPK KO mice. Representative immunoblot of lysate prepared from EDL muscle incubated in the absence (basal) or presence of insulin (30 min) or AICAR (60 min) or AICAR plus insulin (30 min AICAR preincubation followed by 30 min AICAR plus insulin). Phosphorylation of AMPK Thr\(^{172}\), pAkt Ser\(^{473}\), p-mTOR Ser\(^{2448}\), p-rpS6 Ser\(^{235/236}\) and p4E-BP1 Thr\(^{37/46}\) in C57Bl/6 (WT) vs. α2 AMPK KO mice (Panel A) or C57Bl/6 (WT) vs. γ3 AMPK KO mice (Panel B). 40 µg of protein was loaded on each well and equal loading was confirmed by pan-actin antibody.

Figure 2. AMPK and insulin signaling in EDL muscle from α2 and γ3 AMPK KO mice and corresponding wild-type littermates. Phosphorylation of AMPK Thr\(^{172}\) (panel A and B) was determined in EDL muscle obtained from α2 AMPK KO (closed bars), γ3 AMPK KO (hatched bars) and corresponding wild-type (open bars) littermates. Results are mean ± SEM, n = 8 for each genotype. ††genotype effect (p<0.01) within treatment, **treatment effect (p<0.01) within WT. Akt Ser\(^{473}\) phosphorylation (panel C and D) was determined in α2 AMPK KO, γ3 AMPK KO corresponding wild-type (open bars) littermates. Results are mean ± SEM, n = 8 for each genotype. ††main effect of genotype, **effect (p<0.01) of treatment.

Figure 3. AICAR-induced inhibition of mTOR signaling requires functional α2 and γ3 subunits of AMPK. Phosphorylation of S6K1 Thr\(^{349}\) (panel A and B) rpS6 Ser\(^{235/236}\), (panel C and D) and 4E-BP1 Thr\(^{37/46}\), (panel E and F) in EDL muscle from α2 AMPK KO (closed bars) (deleted panel A), γ3 AMPK KO (hatched bars) and corresponding wild-type (open bars) littermates was determined. There is no interaction effect in F. Results are mean ± SEM, n = 8 for each genotype. ††main effect of genotype (p<0.01) and */**treatment effect within genotype (p<0.05/0.01).
Figure 1

A

B

WT  α2 KO  WT  α2 KO

Basal  Insulin  AICAR  AIC  Ins  Basal  Insulin  AICAR  AIC  Ins

pAMPK (Thr-172)
pAkt (Ser-473)
ptmTOR (Ser-2448)
pS6K1 (Thr-389)
prpS6 (Ser-235/236)
p4EBP1 (Thr-37/46)
pan actin
Figure 2

A. Phospho-AMPK Thr-172

B. Phospho-AMPK Thr-172

C. Phospho-Akt Ser-473

D. Phospho-Akt Ser-473
Figure 3

A. Phospho-S6K1-Thr389

B. Phospho-S6K1-Thr389

C. Phospho-rpS6-Ser235/236

D. Phospho-rpS6-Ser235/236

E. Phospho-4E-BP1-Thr37/46

F. Phospho-4E-BP1-Thr37/46