PS1.07 AICAR-induced AMP-activated protein kinase activity in C2C12 myotubes decreases its inhibitory effect on protein synthesis under high glucose supplement 420.00

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***Abstract*— AMP-activated protein kinase (AMPK) monitors cellular energy status and regulates energy metabolism in skeletal muscle. Activation of AMPK and its role modulating protein synthesis under different nutrient availabilities has not been elucidated. Therefore, the purpose of this study was to examine the effect of 5-aminoimidazole-4-carbozamide-1-β -D-ribonucleoside (AICAR) on AMPK amount and activation in C2C12 myotube cultures incubated with low (5 *mM*) or high (25 *mM*) glucose media. Low glucose media increased phosphorylation of AMPK and acetyl-CoA carboxylase (ACC) ($P < 0.05$). Administration of AICAR (2 *mM*) increased ($P < 0.05$) phosphorylation of AMPK and ACC in myotubes regardless of glucose level. Total protein content from cell extract was greater ($P < 0.05$) in myotubes cultured with high glucose media and was further increased ($P < 0.05$) by AICAR treatment. Myotubes cultured with high glucose media possessed 7.5% lower ($P < 0.05$) UbFL-toSV40 ratio compared to those in low glucose media. Administration of AICAR significantly decreased UbFL-to-SV40 ratio by 63% and 54% relative to control in myotubes incubated in low and high glucose media, respectively. Glucose level did not change the phosphorylation level of mammalian target of rapamycin (mTOR). Interestingly, administration of AICAR significantly increased phosphorylation level of mTOR in myotubes cultured with low glucose media but not in those with high glucose media. These data suggest AMPK –activation may regulate protein synthesis/degradation in response to altered glucose availability.**

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***Index Terms*—AMP-activated protein kinase (AMPK), glucose, protein synthesis, mammalian target of rapamycin (mTOR).**

I. INTRODUCTION

AMP-activated protein kinase (AMPK) acts as an energy sensor and its activity is regulated by cellular energy charge. Activity of AMPK can be stimulated by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) without altering cellular levels of ATP, ADP, and AMP [1]. Once activated by metabolic stresses that lower ATP:AMP ratios, AMPK increases energy producing pathways and shuts down energy consuming processes [2].

Protein turnover varies depending on the rate of protein synthesis and degradation [3]. The mammalian target of rapamycin (mTOR) pathway is a key regulator of muscle growth by integrating energy status and growth signalling, thus regulating protein synthesis [4, 5]. Recent studies have shown that AMPK can act as an upstream regulator of mTOR. In particular, activation of AMPK by AICAR administration in rat skeletal muscle suppresses protein synthesis via the inhibition of mTOR through phosphorylation events [6, 7]. These data suggest substantial cross-talk between AMPK and mTOR signaling pathways and provide a mechanism for how cells regulate protein synthesis during periods of energetic stress. Skeletal muscle atrophy involves multiple proteolytic pathways, most notably the ubiquitin-proteasome system, which is largely controlled by cellular ATP levels [8]. AMPK activation by AICAR, which mimics an increase in cellular AMP:ATP ratio, has been shown to stimulate the expression of atrophy-related ubiquitin ligases in C2C12 myotube cultures [9]. However, it is unknown whether chronic activation of AMPK under different nutrient condition alters the activation of mTOR signaling and ubiquitin-proteasome system. Here, we investigated the function of forced AMPK activation by AICAR on mTOR phosphorylation and protein synthesis in C2C12 myotubes incubated in media with different levels of glucose.

II. MATERIALS AND METHODS

Cell culture: C2C12 mouse muscle cells were plated at 40,000 cells/ml in 12 well plate and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and antibiotics (1% antibiotic antimycotic solution [Sigma] and 0.1% getamycin [Gibco]) at 37 ºC and 5% CO_2. At 80% confluence, C2C12 cells were switched to media containing 2% heat-inactivated horse serum in DMEM with antibiotics (differentiation media; DM). After 5 d of differentiation, fused myotubes were treated with 2 *mM* AICAR in medium containing either low (5

mM) or high (25 *mM*) glucose.

Protein assay: After 24 h in treatment medium, C2C12 myotubes were homogenized in ice cold RIPA buffer containing 50 *mM* Tris-HCL, 150 *mM* NaCl, 1 *mM* EDTA, 1 *mM* PMSF, 0.25% sodium deoxycholate, 1% NP-40, 1 *mM* Na_3VO_4, 1 *mM* NaF, and phosphatase inhibitor cocktail 1 and 2 (Sigma). Samples were sonicated on ice for 5 sec and centrifuged for 10 min at 10,000 g at 4 ºC. Protein concentration was determined using a BCA Protein Assay kit according to the manufacturer's protocol (Pierce).

Proteasome activity: When C2C12 cells reached to 80% confluence, cells were co-transfected with 26S proteasome activity reporter plasmid (UbFL-luc) containing firefly luciferase and plasmid expressing Renilla luciferase (pRL-SV40) using Lipopectamine 2000 (Invitrogen). After 24 h, media were changed to DM. After AICAR and glucose treatment, luciferase activity in cell lysates was determined with a luciferase assay kit (Promega). Luciferase values were normalized to total cell protein.

Western blotting: Thirty micrograms of protein were resolved by SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes and membranes were blocked, and immunoblotted with primary antibodies specific for acetyl CoA-carboxylase (ACC), phospho-ACC, AMP-activated protein kinase (AMPK), phospho-AMPK, mammalian target of rapamycin (mTOR), phospho-mTOR, or α-tubulin. Secondary antibodies conjugated with horseradish peroxidase (1:1000) were applied for 1 h and bands were visualized using enhanced chemiluminescence (ECL; Amersham) and quantified using NIH ImageJ.

Statistics: Data are reported as mean ± s.e. and compared with Student's *t* test. Values were considered significant at the $P < 0.05$ level.

III. RESULTS AND DISCUSSION

We utilized an AMP analog, AICAR, to activate AMPK and determine its role on regulating mTOR signaling and ubiquitin-proteasome activity under different environmental level of energy availability (glucose). As shown in Figure 1, phosphorylation of AMPK was greater ($P < 0.05$) in myotubes cultured in low glucose-containing media. These data are consistent with the previous studies showing that decreased ATP level by glucose restriction activates AMPK [10]. Administration of AICAR stimulates AMPK phosphorylation in C2C12 myotubes regardless of glucose level in cell culture media (Figure 1). Interestingly, however, AICAR increases AMPK phosphorylation even in high glucose media (Figure 1). Once absorbed by the cell, AICAR is metabolized into ZMP, which acts as a metabolic activator of AMPK without altering the [AMP], [ADP], and [ATP] within the cell. This suggests AMP (in this case, ZMP) may have higher binding affinity to cystathionine beta synthase domains in AMPK γ subunit than ATP or increased ZMP level helps the function of upstream kinase(s) to phosphorylate AMPK. Alternatively, AMPK phosphorylation may remain longer by lowering protein phosphatase activity by the function of ZMP in blocking protein phosphatase activity. However, the function of AICAR on inhibiting the specific protein phosphatase activity remains unknown.

Phosphorylation of AMPK was confirmed by ACC phosphorylation (Figure 2). Myotubes in low glucose media had greater ($P < 0.05$) levels of ACC phosphorylation compared to those in high glucose media. Even so, administration of AICAR further increased ACC phosphorylation independent of glucose level (Figure 2). Although we did not assess the rate fatty acid oxidation in this study, an increase in ACC phosphorylation in myotubes incubated with low glucose media is consistent with the well-known reverse Randle cycle, where elevated glucose uptake into cytosol decreases fatty acid oxidation. Further investigation is necessary to define precisely actual glucose and fatty acid uptake and oxidation by the cultures.

Total protein content from cell extracts was increased ($P < 0.05$) in C2C12 myotubes incubated with high glucose media (Figure 3). This is consistent with previous studies showing that added energy stimulates muscle protein synthesis in vivo [11] and in vitro [12] independent of insulin. Administration of AICAR further increased ($P < 0.05$) protein content in high glucose supplement (Figure 3). However, this increase in protein content in cells treated with AICAR conflicts with the aforementioned understanding that AMPK activation inhibits protein synthesis. This is especially true given previous studies have reported that AICAR-induced AMPK activation stimulates myofibrillar protein degradation in C2C12 myotubes [9]. To that end, we evaluated ubiquitin proteasome activity in these cells using a reporter plasmid (UbFL) containing firefly luciferase and plasmid expressing Renilla luciferase (SV40). Myotubes incubated with high glucose media showed 7.5% lower ($P < 0.05$) UbFL-to-SV40 ratio compared to those in low glucose media representing decreased ubiquitin-proteasome activity in myotubes cultured with high glucose media (Figure 4; comparison of AICAR-untreated controls). Administration of AICAR decreased ($P < 0.05$) UbFL-to-SV40 ratio by 63% and 54% relative to control in myotubes incubated in low and high glucose media, respectively. This suggests

that high glucose somehow decreases proteasome activity or protects protein degradation, thus increasing protein accretion.

Furthermore, AICAR decreased ($P < 0.05$) phosphorylation of mTOR in myotubes cultured with low glucose media but did not have alter mTOR phosphorylaton in myotubes cultured in high glucose-containing media (Figure 5). This may be partly explained by recent studies showing glucose induces Akt activity, thereby stimulating the mTOR pathway by decreasing the AMP/ATP ratio and thus preventing AMPK from inhibiting mTOR [13]. However, this does not explain why AICAR was able to induce AMPK activity even in high glucose media in our studies. Results of these studies suggest the existence of other regulatory mechanisms that may regulate anabolic processes when cells have adequate energy charge.

IV. CONCLUSION

AICAR stimulates AMPK phosphorylation in muscle cell cultures regardless of glucose content in the media. Moreover, protein content is increased in myotubes cultured in high glucose. Yet, activation of AMPK further increases protein content in the presence of high glucose-containing media. This could be due, in part, that higher glucose availability (energy) protects cells against protein degradation, perhaps by blocking the inhibitory effect of activated-AMPK on protein synthesis pathway. These data suggest other regulatory mechanisms, or signaling pathways exist that regulate cellular protein synthesis events in a hierarchical fashion when cells have excessive energy charge.

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Figure 1. Effect of AICAR (2 *mM*) on phosphorylation of AMPK in C2C12 myotubes incubated (24 h) with low (5 *mM*) or high (25 *mM*) glucose media. Blots were incubated with antibodies specific for phospho-AMPK and then stripped and re-incubated with AMPK antibodies. Data are means ± S.E. of 3 samples per group. Means bearing different letters differ (P < 0.05).

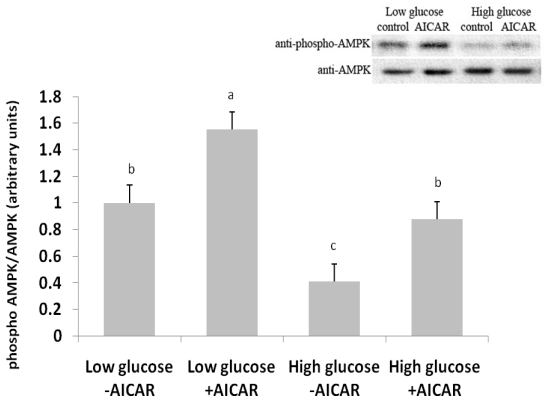
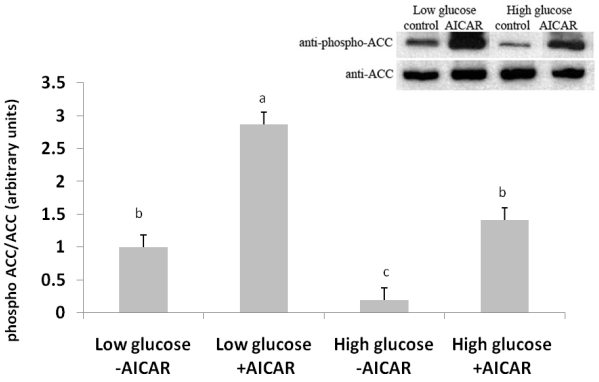
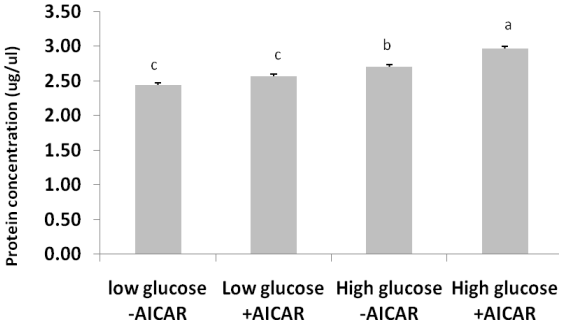


Figure 2. Effect of AICAR (2 *mM*) on phosphorylation of ACC in C2C12 myotubes incubated (24 h) with low (5 *mM*) or high (25 *mM*) glucose media. Blots were incubated with antibodies specific for phospho-ACC and then stripped and re-incubated with ACC antibodies. Data are means ± S.E. of 3 samples treatment



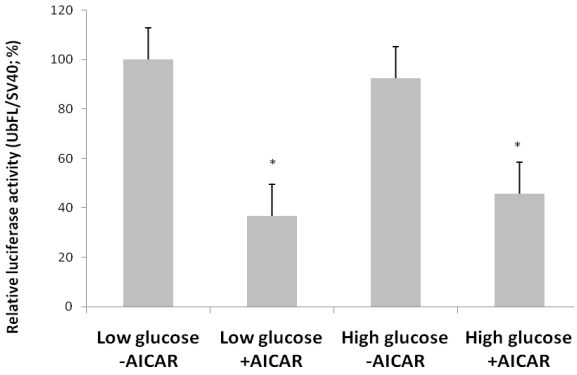
combination. Means bearing different letters differ (P < 0.05).

Figure 3. Effect of AICAR (2 *mM*) on total protein concentration from cell extracts of myotubes incubated (24 h) with low (5 *mM*) or high glucose (25 *mM*) media. Data are means ± S.E. of 3 samples per treatment



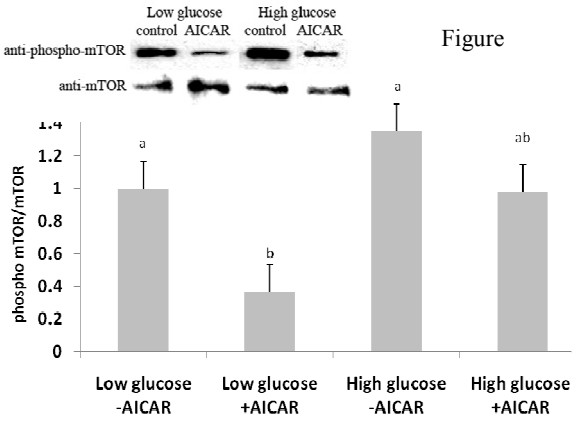
combination. Means bearing different letters differ (P < 0.05).

Figure 4. Effect of AICAR (2 *mM*) and low (5 *mM*) and high high (25 *mM*) glucose on ubiquitin protreasome activity in muscle cell cultures. C2C12 myotubes were co-transfected with 26S ubiquitin proteasome activity reporter plasmid (UbFL-luc) containing firefly luciferase



and plasmid expressing Renilla luciferase (pRL-SV40) to assess transfection efficiency. A reduction in the UbFL-to-SV40 ratio represents an increase in proteasome activity. Astericks indicated significance of treatment combination (P < 0.05) versus other groups (n=6 per treatment combination).

Figure 5. Effect of AICAR (2 *mM*) on phosphorylation of mTOR in C2C12 myotubes incubated with low (5 *mM*) or high (25 *mM*) glucose media. Blots were incubated with antibodies specific for phospho-mTOR and then stripped and re-incubated with mTOR antibodies. Data are means ± S.E. of 6 samples per treatment combination. Means bearing different letters differ (P < 0.05).



Figure