AMPK'S INHIBITION OF mTOR ACTIVITY MEDIATED BY INTRACELLULAR ENERGY STATUS

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***Abstract*—AMP-activated protein kinase (AMPK) activation has been shown to decrease protein synthesis by inhibition of the mTOR pathway using various chemical activators. However, two AMPK activators manipulate intracellular energy status by different means. Administration of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) results in accumulation of the AMP analogue, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl monophosphate while β-guanidinopropionic acid (β-GPA) activates AMPK by decreasing ATP levels. The objectives of this study were to define the dose of β-GPA necessary to activate AMPK to similar levels as AICAR and to assess the ability of these two compounds to modulate mammalian target of rapamycin (mTOR)-mediated protein synthesis. Exposure to 25 mM β-GPA resulted in AMPK activation equivalent to exposure to 0.5 mM AICAR. However, a greater decrease ($P < 0.05$) in mTOR phosphorylation at Ser2448 in cells treated with 25 mM β-GPA was observed compared to those treated with AICAR. These data suggest energy status, particularly ATP availability, may play a larger role in the inhibition of mTOR and protein synthesis than AMPK activation.**

***Index Terms*—AMPK, mTOR, protein synthesis.**

I. INTRODUCTION

Skeletal muscle must adapt to fluctuating energy availabilities. To this end, rapid and efficient coordination of metabolic pathways is necessary to preserve ATP for optimal function and cellular homeostasis. During contraction, ATP utilization can increase by as much as 100-fold. Breakdown of ATP yields ADP and inorganic phosphate; in turn, two ADP can be used by myokinase to generate ATP and AMP. Because AMP increases correspond with decreases in ATP, the AMP:ATP ratio is a particularly sensitive indicator of cellular energy charge. In response to increases in intracellular AMP:ATP, the "master" energy sensor, AMP-activated protein kinase (AMPK) is capable of regulating multiple pathways responsible for energy production and consumption.

AMPK is a heterotrimeric protein, composed of an α, β, and γ subunit. The regulatory γ-subunit contains domains capable of binding the allosteric activator, AMP. Because AMP competes with ATP for the binding sites, AMPK is able to detect changes in AMP:ATP. Binding of AMP induces a conformational change, making AMPK a better substrate for upstream kinases for phosphorylation of the α-subunit while also preventing dephosphorylation by protein phosphatases (Scott, et al., 2004).

Once activated, AMPK phosphorylates a host of metabolic enzymes that typically result in an increase in fat, protein, and carbohydrate catabolism while simultaneously blunting anabolic processes unnecessary for survival (reviewed by: Witczak, Sharoff & Goodyear, 2008). As part of its energy conserving response, AMPK activity decreases protein synthesis via indirect inhibition of the mammalian target of rapamycin (mTOR) pathway. MTOR is activated via phosphorylation at Ser4228 by the upstream kinase, Akt. In response to Akt phosphorylation, mTOR activates 70-kDA ribosomal protein S6 kinase (p70-S6K) and inhibits the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1), thereby increasing protein synthesis. (Bolster, Crozier, Kimball & Jefferson, 2002). Activation of AMPK inhibits mTOR activity by activating tuberous sclerosis protein 2 (TSC2), and by preventing the formation of the functional mTOR complex (Gwinn, et al., 2008). Conversely, AMPK activity increases in skeletal muscle experiencing increased protein synthesis due to overload-induced hypertrophy (Thomson and Gordon, 2005). Further, pigs that possess constitutively active AMPK due to a naturally occurring mutation in the γ-subunit ($\gamma3^{R225Q}$), do not exhibit reduced lean muscle growth (Carr, Morgan, Berg, Carter & Ray, 2006). Additionally, AMP and ATP levels in AMPK $\gamma3^{R225Q}$ pigs are similar to wild type pigs (Copenhafer, Richert, Schinckel, Grant & Gerrard, 2006), suggesting that adenine nucleotide levels may play a more significant role in regulating protein synthesis than AMPK activity.

Interestingly, two chemical activators of AMPK have contrasting effects on ATP levels. The AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), is taken up by cells and metabolized to the AMP analogue, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl monophosphate (ZMP) (Corton, Gillespie, Hawley & Hardie, 1995). Accumulation of ZMP mimics an increase in the AMP:ATP ratio, thereby activating AMPK without affecting ATP levels in the cell (Hayashi, Hirshman, Fujii, Habinowski, Witters & Goodyear, 2000). Conversely, chronic treatment with β-guanidinopropionic acid (β-GPA), a creatine analog, results in a marked reduction of ATP in muscle (Zong, et al., 2002). While treatment with either of these chemicals results in AMPK activation, this activation is accompanied by different ATP levels. Our objectives were to determine the dose of β-GPA necessary to activate AMPK to similar levels as AICAR, and to evaluate the effect of these compounds on protein synthesis, indicated by

mTOR activation. We hypothesized that despite similar AMPK activation, mTOR activation would be greater in cells treated with AICAR due to improved energy (ATP) status.

II. MATERIALS AND METHODS

A. Cell Culture

C_2C_{12} myoblasts were plated at a density of 5,000 cells/cm^2 and grown in Dulbecco's modification of Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Sigma), and antibiotics [1% antibiotic/antimycotic solution (Sigma) and 1% gentamycin (Gibco)] at 37°C and 5% CO_2. At 90% confluence, cells were induced to differentiate with 2% horse serum in DMEM with antibiotics. After differentiation for five days, cells were treated with 0.5 mM AICAR or varying concentrations of β-GPA (0, 0.5, 1, 10, 25, or 50 mM) in the differentiation-inducing media for 24 h.

B. Western blot analysis

Myotubes were harvested and homogenized in ice-cold RIPA buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM NaF, and 1 µg/ml aprotinin, leupeptin, and pepstatin with phosphatase inhibitor cocktails 1 and 2 (Sigma, St. Louis, MO, USA)] and centrifuged for 10 min at 10,000 *g* at 4°C. Protein concentration was determined using BCA Protein Assay kit (Pierce, Rockford, IL, USA). Then, 30 µg protein per sample was resolved by SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes, blocked, and immunoblotted with primary antibodies specific for AMPK, phosphorylated AMPK, mTOR, phosphorylated mTOR, and α-tubulin. Secondary antibodies conjugated with Dylight fluorophores (Thermo Scientific, Waltham, MA, USA) were applied for 2 h and membranes were visualized and quantified using LI-COR® Odyssey® Infrared Imager (Lincoln, NE, USA). All primary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA).

C. Statistical analysis

Data are presented as means ± SE. Differences in means were estimated using ANOVA followed by Student's *t* comparison of means test in SAS JMP (Cary, NC, USA). Differences were considered statistically significant at $P < 0.05$.

III. RESULTS AND DISCUSSION

A. AICAR vs. β-GPA mediated activation of AMPK

Administration of 0.5 mM AICAR resulted in a 54.3% ± 4.5 increase ($P>0.05$) in phosphorylation of total AMPK. Phosphorylation of AMPK due to β-GPA administration was dose dependent, with 25mM β-GPA phosphorylating AMPK to approximately the same level (54.1% ± 4.5) as 0.5 mM AICAR.

B. AMPK and mTOR phosphorylation

While we were able to phosphorylate AMPK to the same levels with AICAR and β-GPA, we observed a decrease in mTOR phosphorylation at Ser2448 only in cells exposed to β-GPA (Figure 2). This decrease in mTOR phosphorylation was dose dependent and inversely correlated with AMPK activity ($R^2=0.75$, $P<0.05$). These data suggest that AMPK's inhibition of mTOR phosphorylation by Akt may be mediated by intracellular ATP levels. These results concur with studies showing pigs possessing the RN mutation do not suffer from decreased lean muscle growth.

Previous research has shown that AICAR induced AMPK activation synergistically increased IGF-1 activation of Akt, the upstream kinase of mTOR (Tong, Yan, Zhu & Du, 2009), supporting the hypothesis that the effects of AMPK activation are capable of manipulation. Additionally, the α1 subunit of AMPK is highly activated in skeletal muscle undergoing hypertrophy while α2 activity is not altered, suggesting differential responses of AMPK dependent upon the composition of the heterotrimer (McGee, Mustard, Hardie & Baar, 2008).

IV. CONCLUSION

Using a cell culture model, we show that mTOR activation can be modulated by method of AMPK activation. These results imply that intracellular ATP levels may play a larger role in the regulation of protein synthesis than AMPK activity. These findings suggest that AMPK, under the proper conditions, could serve as a potential target for increasing lean muscle growth in livestock. While the exact mechanisms behind this effect are still unknown, we suspect crosstalk

between AMPK and Akt may be involved. Additionally, the role of the α subunit isomers in AMPK activity during periods of energy depletion are not fully understood and may contribute to our observed response.

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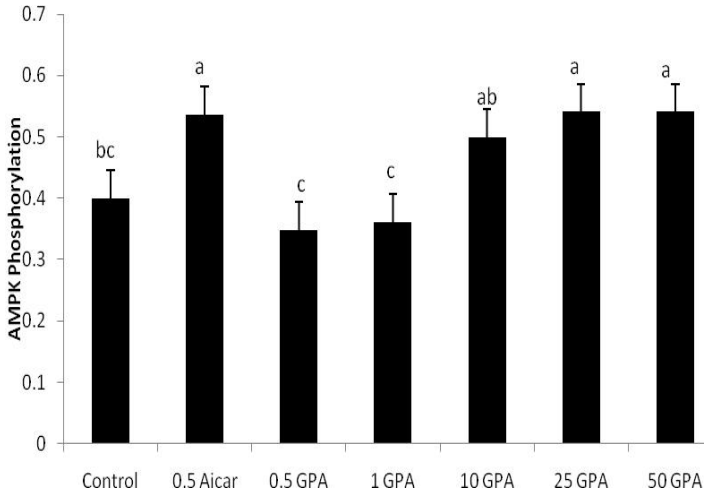

Figure 1. AMPK phosphorylation (phosphorylated/ total) in response to varying concentrations (mM) of β-GPA or AICAR (n = 3 per treatment). Average band intensity from a single western blot. Different letters indicate significance ($P < 0.05$).

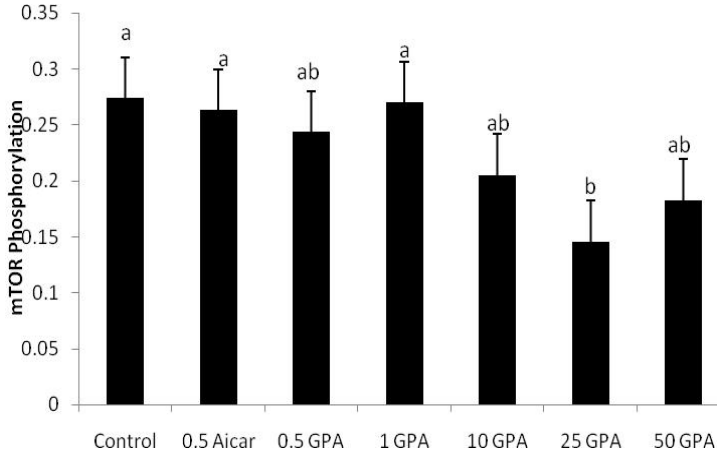

Figure 2. mTOR phosphorylation (phosphorylated/ total) in response to varying concentrations (mM) of β-GPA or AICAR (n = 3 per treatment). Average band intensity from a single western blot. Different letters indicate significance ($P < 0.05$).