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Original Article

Sepsis and AMPK Activation by AICAR Differentially Regulate FoxO-1, -3 and -4 mRNA in Striated Muscle

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Abstract: Although much is known regarding the posttranslational regulation of the FoxO transcription factors, there is little appreciation of how stressors which regulate cellular energy status effect the various FoxO family members at the mRNA level. The hypothesis of the present study was that exposure of differentiated muscle cells to agonists of AMP-activated protein kinase (AMPK) would increase the mRNA content of various FoxO mRNA transcripts. Stimulation of AMPK in vivo by the injection of AICAR into mice increased FoxO1 and FoxO3 (but not FoxO4) mRNA in skeletal muscle. A comparable increase in these FoxO mRNAs was seen in skeletal muscle in response to sepsis which also increased AMPK phosphorylation. In contrast to the in vivo data, FoxO1, 3 and 4 mRNA content was decreased dose-dependently, with the decrement in FoxO1 being the largest, in C_2C_{12} myotubes incubated with the AMPK agonists AICAR or metformin. Treatment of myotubes with 2-deoxyglucose or reducing the media glucose concentration also decreased mRNA content for FoxO1 and FoxO4. All stressors increased AMPK phosphorylation under in vitro conditions. Incubation of myotubes with AICAR decreased the rate of protein synthesis and increased protein degradation. Finally, treatment with the AMPK inhibitor compound C prevented both the AICAR-induced changes in FoxO mRNA and changes in protein metabolism. Our data indicate FoxO mRNA expression is down-regulated by AMPK activation and energy depletion in cultured myotubes, but that a contrasting increase in FoxO1 and FoxO3 mRNA is observed in vivo with the agent (and in response to sepsis) suggesting the expression of these FoxOs may be controlled by other hormonal or energy sensing cues under in vivo conditions.

Key Words: Sepsis, energy stress, protein synthesis, muscle

Introduction

The Forkhead or Fox box class 'Other' (FoxO) proteins are evolutionarily conserved genes controlling cell cycle, apoptosis, and the stress response [1-4]. Additionally, the FoxO proteins integrate signals related to nutrient availability and environmental cues to regulate energy metabolism in a variety of tissues, including muscle, liver and adipose tissue [3]. The FoxO subfamily of transcription factors - in humans FoxO1 (FKHR1), FoxO3 (FKHRL1), and FoxO4 (AFX) - is traditionally recognized as down stream targets of the protein kinase Akt [4]. The phosphorylation of FoxO results in their nuclear exclusion and renders them transcriptionally inactive. The cytoplasmic localization of the FoxO proteins accelerates their ubiquitinization and ultimate degradation [5]. In addition, a number of posttranslational modifications of the FoxO proteins have been described which are capable of regulating their function [2]. Hence, FoxO factors are generally considered to be regulated at three levels: transcriptional activity, localization, and stability.

Alternatively, FoxO activity may also be regulated at the level of FoxO gene expression and it has been posited that regulation of FoxO mRNA content may be as important as its phosphorylation in controlling muscle metabolism [6]. However, the constitutive expression pattern as well as the ability of various physiological mediators to differentially regulate individual FoxO mRNAs suggests their physiological role(s) may be unique [2,7]. For example, overnight fasting, exercise, diabetes, uremia and cancer have been reported to increase FoxO1 and Foxo3 mRNA and protein

in skeletal muscle, with little or no change in FoxO4 expression [8-10]. However, these effects may be tissue-specific because diabetes decreases the hepatic content of FoxO1 and FoxO3 mRNA [11]. Mechanistically, an increase in FoxO3 protein is capable of regulating protein balance in skeletal muscle by increasing the activity of the muscle-specific ubiquitin E3 ligase atrogin-1, whereas a dominant-negative FoxO3 prevents starvation-induced muscle atrophy [12,13]. Hence, understanding other FoxO regulating mechanisms may be of importance in muscle during stress conditions where protein balance is disturbed.

One mechanism by which cells sense their energy status is via the heterotrimeric serine (Ser)/threonine (Thr) kinase referred to as AMP activated protein kinase (AMPK) [14]. AMPK is an essential intermediate in the control of fundamental cellular processes such as growth, proliferation, and survival [15-17]. Additionally, AMPK orchestrates multiple signaling pathways controlling nutrient uptake and fuel metabolism [14,18,19]. In response to energy-depleting stressors which increase the cellular AMP/ATP ratio, AMPK acts to balance energy consumption with energy production by suppressing ATP-expensive and activating ATP-repleting processes. Activation of AMPK by 5-aminoimidazole-4-carboxamide riboside (AICAR) has been reported to decrease muscle protein synthesis [20-22] and increase the muscle-specific ubiquitin ligases atrogin-1 and MuRF1 thereby enhancing proteolysis [23,24]. In general, AMPK induces these and other effects at a cellular level primarily through two means: direct phosphorylation of rate-limiting or otherwise strategic components involved in pathways of metabolic control and through a less-well understood control of gene expression.

The outcome of AMPK activation on the expression of specific genes is, in general, consistent with the corresponding metabolic responses and tissue adaptations observed after acute and chronic activation of the kinase. The preponderance of data has focused on either the repression of gluco-neogenic and lipogenic genes in the liver [25-27], or the induction of genes involved in mitochondrial biogenesis, glucose and lipid metabolism, and glucose transport in skeletal muscle [28-30]. However, whether AMPK or energy stress alters FoxO mRNA expression in muscle, remains largely unexplored. Despite ample evidence demonstrating the FoxOs are contributing factors to the (patho)physiological response of skeletal muscle to many atrophic stimuli, a complete understanding of the initiating stimuli and cellular signaling underlying their regulation is still lacking. Given the pivotal role played by AMPK in the adaptive responses to energy insufficiency it is reasonable to suspect its activation may be capable of regulating the expression of one or more of these genes. Therefore the purpose of these studies was to examine changes in the mRNA content of specific FoxOs that have been implicated in atrophy in response to AMPK activation and sepsis using both in vitro and in vivo experimental approaches.

Materials and Methods

Cell culture

C_2C_{12} myoblasts (American Type Culture Collection, Manassas, VA) were maintained in Eagle Minimum Essential Media (EMEM) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin (250 ng/ml) (all from Mediatech, Herndon, VA) under 5% CO_2 at 37°C, exactly as previously described [23]. For experimental treatments myoblasts were subcultured into 6-well tissue culture plates (Grenier Bio-One, Frickenhausen, Germany). At ~100% confluence the cells were switched to media consisting of EMEM with the above antibiotics/antimycotics and 10% bovine calf serum (Hyclone, Logan, UT) to promote myoblast fusion and differentiation to myotubes. Cells were allowed to differentiate for 4 days prior to experimental manipulation. Myotubes were provided with fresh differentiation media for 2 hours immediately preceding treatment on the 5th day. All experiments were performed using serum-free EMEM plus antibiotics/antimycotics. Myotubes were incubated with 5-aminoimidazol-4-carboximide ribonucleoside (AICAR; Toronto Research Chemicals, Ontario, Canada), 1-1 dimethylbiguanide hydrochloride (metformin), 2-deoxy D-glucose (2-DG), D-mannitol, and/or compound C (all from Sigma-Aldrich, St. Louis, MO) at concentrations and times specified in the figures and text. The specific doses and time points used in the current study have been optimized based on previously published results [23]. In preliminary studies, cell viability

was assessed by determining lactate dehydrogenase (LDH; Sigma-Aldrich) in the culture media of cells incubated with various doses of AICAR and metformin for 24 h. The LDH release data are expressed as a percent of vehicle treated time-matched control values.

Animals

C57BL/6 male mice were obtained from Charles Rivers Laboratories (Wilmington, MA). All mice were housed in a controlled environment and provided water and standard rodent chow (Harlan Teklad, Indianapolis, IN) ad libitum for 1 wk before use. At the time of the study, mice were 8-9 wk of age and weighed 22-23 g. All experiments were performed in adherence with the National Institutes of Health "Guide for Care and Use of Laboratory Animals" and with the approval of The Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. For the first study, on the morning of the study, mice were injected intraperitoneally (IP) with AICAR (1 mg/kg body weight; 0.5 ml/mouse) or an equivalent volume of isotonic saline. Six hours after the injection of AICAR or saline, mice were anesthetized with ketamine-xylazine (90 and 9 mg/kg, respectively), and the gastrocnemius/plantaris complex and heart (ventricular muscle only) were excised and frozen in liquid nitrogen. In the second study, male mice were anesthetized with ketamine-xylazine and a midline line laporatomy performed. Sepsis was induced by cecal ligation and puncture (CLP), as previously described [31], which produces a clinically relevant model of peritonitis. Mice in this study were sacrificed after 24 h, as described above, and skeletal and cardiac muscle sampled. All mice were pair-fed to match food consumption of the appropriate control group. These time points were selected based on preliminary studies indicating optimal activation (e.g., phosphorylation) of AMPK in skeletal muscle.

Multi-probe template production for RNAse protection assay (RPA). Primer selection for mouse genes of interest was determined with the help of Genefisher software [32]. The lengths of amplified regions were chosen to allow distinct resolution during electrophoretic separation. Primers were synthesized (IDT, Coralville, IA) with restriction sites for EcoRI or KpnI at the 5' end and with three extra bases at the extreme 5' end as follows: FoxO1–Forward (5'-GCA GAA TTC CCT AAT TCG GTC ATG CCA GCG TA-3') and Reverse (5'-GCA GGT ACC CCG AAT GAT GGA CTC CAT GTC ACA-3'); FoxO3 - Forward (5'-GCA GAA TTC GCT GGC CGA GAA CCT CAT GGA -3') and Reverse (5'-GCA GAA TTC GCT GGC CGA GAA CCT CAT GGA-3'); FoxO4 - Forward (5'-GCA GAA TTC GAG GCC GGA GTC TGA AGT GCT A -3'), Reverse - (5'-GCA GGT ACC AAG CCG GAG AGA CCG CTC CTA-3'); and L32 - Forward (5'- GCA GAA TTC CGG CCT CTG GTG AAG CCC AA-3'), Reverse (5'-GCA GGT ACC CCT TCT CCG CAC CCT GTT GTC A-3'). PCR was conducted using HotStarTaq DNA Polymerase (Qiagen, Valencia, CA) and mouse total RNA reverse-transcribed with SuperscriptTM First- Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR products were phenol:chloroform extracted, ethanol precipitated, and sequentially digested with KpnI and EcoRI (Promega, Madison, WI). Digested products were gel-purified, re-extracted, and cloned into KpnI/EcoRI-digested pBluescript II SK+ (Stratagene, La Jolla, CA). Plasmid DNA was isolated with both QIAprepR Spin Miniprep and Plasmid Maxi Kits (Qiagen). Plasmids with inserts were verified by sequencing in the Pennsylvania State College of Medicine Molecular Genetics Core Facility. Final constructs were linearized with EcoRI, gel-purified, and quantitated spectrophotometrically.

RNA extraction and RPA

Total RNA was extracted from cells using TRI Reagent (Molecular Research Center, Cincinnati, OH) and the mRNA content was determined by RPA. An aliquot (2 μl) of template was prepared using T7 Polymerase with buffer (Fermentas, Hanover, MD), NTPs and tRNA (Sigma-Aldrich), RNaisin and DNase (Promega), and ^{32}P-UTP (Amersham Biosciences, Piscataway, NJ). Unless otherwise noted, the entire RPA procedure including labeling conditions, component concentrations, sample preparation, and gel electrophoresis was as published (BD Pharmingen, San Diego, CA). Hybridization buffer was 80% formamide and 20% stock buffer (200 mM Pipes pH 6.4, 2 M NaCl, and 5 mM EDTA). Hybridization proceeded overnight at 56°C in a dry bath incubator (Fisher Scientific, Pittsburgh, PA) without the use of mineral oil. Samples were treated with RNAse A+T1 (Sigma) in 1x RNAse buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, and 300 mM NaCl) followed

by Proteinase K (Fisher Scientific) in 1x Proteinase K buffer (50 mM Tris pH 8.0, 1 mM EDTA, 1% Tween-20). Following ethanol precipitation, samples were resuspended in 5 µl of loading buffer (98% formamide (v/v), 0.05% xylene cyanol (w/v), 0.05% bromphenol blue (w/v), and 10 mM EDTA). Polyacrylamide gels were run in an S3S Sequencing System (Owl Separation Systems, Portsmouth, NH), transferred to chromatography paper, and dried for 10 minutes at 80°C (FB GD 45 Gel Dryer, Fisher Scientific). Gels were exposed to a PhosphorImager screen (Molecular Dynamics Inc., Sunnyvale, CA). Data were visualized and analyzed using ImageQuant software (Version 5.2, Molecular Dynamics). Signal densities for mRNAs were analyzed in the linear range and normalized to L32 mRNA.

Immunoblot analysis

Following drug treatment cells were rinsed with cold Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen) and collected on ice in lysis buffer (20 mM HEPES, 50 mM β-glycerophosphate, 1% Triton X-100, 100 mM KCl, 2 mM EDTA, 50 mM NaF, 1 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 1 mM sodium orthovanadate, and 2 µg/ml leupeptin). Lysates were then passed several times through a 27 gauge needle and centrifuged at 1500 g for 10 minutes at 4°C. A portion of the resulting cell supernatant was used to determine protein concentration via a bicinchoninic acid assay kit (Pierce; Rockford, IL).

Samples were loaded according to total protein content (20 µg) on polyacrylamide gels for separation by SDS-PAGE. Proteins were transferred to PVDF membrane (Biotrace; PALL, Pensacola, FL), blocked in nonfat dry milk, and incubated overnight at 4°C with phosphospecific antibodies for AMPKα (Thr172) and acetyl-CoA carboxylase (ACC; Ser79) (both from Cell Signaling Technology, Beverly, MA), as previously described [23]. Excess primary antibody was removed by washing in TBST (1X TBS + 0.1% Tween 20) and membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Sigma-Aldrich) at room temperature. Blots were developed using enhanced chemiluminescence (ECL; Amersham Biosciences) in accordance with the manufacturer's instructions and exposed to BioMax XAR X-ray film (Kodak, Rochester, NY) in a cassette equipped with a DuPont Lightning Plus intensifying screen. Developed film was scanned (ScanMaker IV; Microtek USA, Carson, CA). Following development, antibody was removed from membranes by treatment with a solution containing 62.5 mM Tris, pH 6.8, 2% (weight/volume) SDS, and 100 mM β-mercaptoethanol in a 50°C water bath for 15 minutes. Blots were then blocked with nonfat dry milk and incubated overnight at 4°C with antibodies for AMPKα and ACC (both from Cell Signaling Technology). An antibody against β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) served as a control for equal protein loading of samples. Membranes were then processed as above.

Protein synthesis and degradation

C_2C_{12} myotubes were cultured as described above and stimulated with either AICAR (2 mM) or compound C (20 mM) + AICAR (2 mM) for 16 h. For measurement of protein synthesis, cells were then labeled with 2 µCi/well of [^{3}H]- phenylalanine (132 Ci/mmol, Amersham, Arlington Heights, IL) for 4 h. Cells were washed twice with MEM, isolated, and cell protein was precipitated overnight at 4°C in a final concentration of 10 trichloroacetic acid (TCA). Pellets were washed three additional times with 10% TCA, solubilized in 2 N sodium hydroxide and counted in Scint-safe™ liquid scintillation cocktail.

For protein degradation, myotubes were pulse labeled with for 48 with [^{3}H]-L-tyrosine (2 µCi/well). Some cells were collected at this time (pulse cells), whereas for other cells, the radiolabeled medium was removed and replaced with fresh medium lacking radioactivity (chase). Cells were then chased for 24 h in the absence (control) or presence of AICAR or metformin. Cells were collected and precipitated in 10% TCA, and the TCA precipitable counts determined.

Statistical analysis

Results for individual experiments using C_2C_{12} cells were replicated in 3-5 independent experiments whereas all in vivo studies were performed on two separate occasions. All data are presented as means ± SE calculated from the pooled data. Data were analyzed by ANOVA

with a Student-Neuman-Keuls post – test to determine treatment effect when ANOVA indicated a difference among the means. Differences between groups were considered significant when $P < 0.05$.

Results

In vivo regulation of FoxO mRNAs

Initial studies were performed to determined whether sepsis, which is known to induced cellular energy stress, altered muscle FoxO mRNA content. Sepsis increased the gastrocnemius mRNA content for FoxO1 and FoxO3 by approximately 2.5- to 3-fold (**Figure 1**). No change in FoxO4 mRNA was detected in gastrocnemius in response to sepsis. In contrast to skeletal muscle, sepsis did not alter the mRNA expression of any of the FoxOs in cardiac muscle at the 24-h time point. A similar trend for sepsis to increase FoxO1 and Fox3 in gastrocnemius was also seen 6 h after induction of sepsis but these changes, which averaged 40-60% above control values, failed to achieve statistical significance (data not shown). Likewise, at this earlier 6-h time point, there was no sepsis-induced change in the mRNA content for any of the FoxOs in cardiac muscle (data not shown).

To confirm that sepsis altered muscle energy status, we assessed the phosphorylation of the α-subunit of AMPK at the Thr 172 residue, a signaling event requisite for nearly all AMPK activity [33], and the phosphorylation of its downstream substrate ACC. As illustrated by the representative Western blot in **Figure 1**

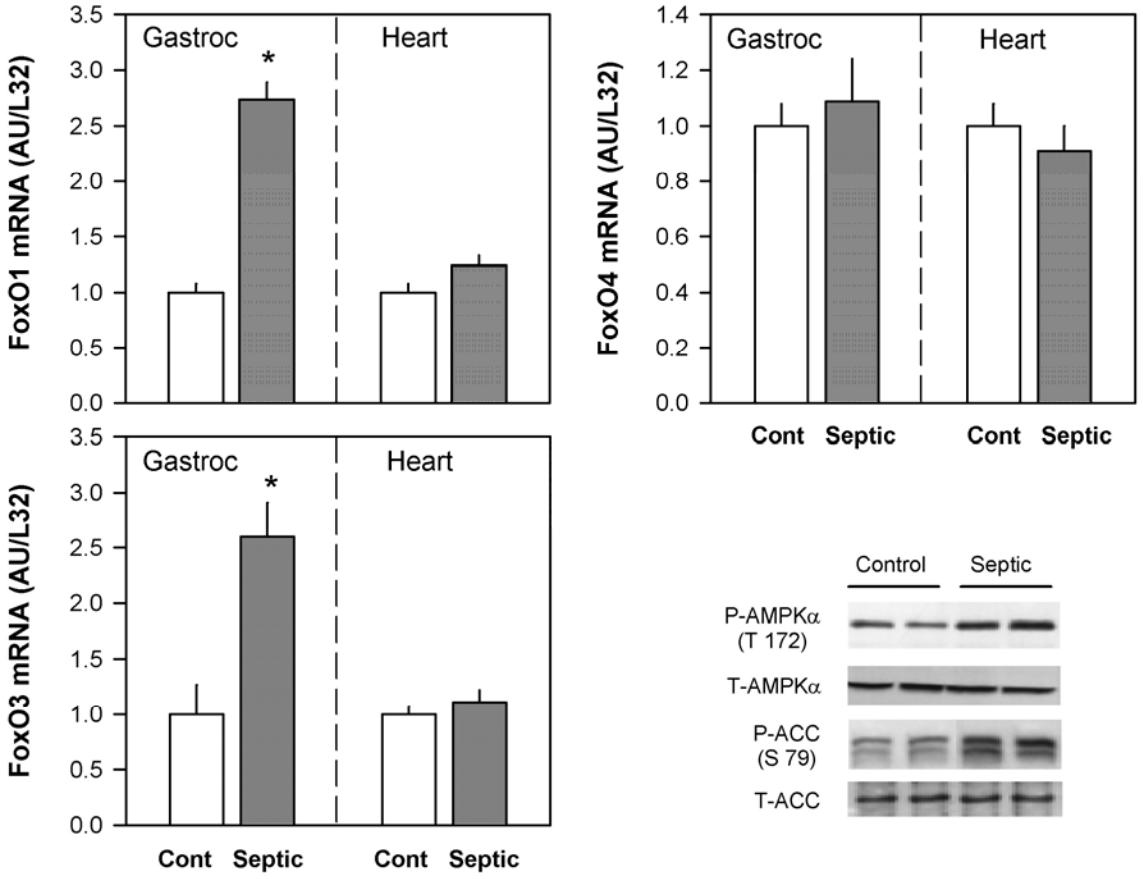

Figure 1. In vivo regulation of FoxO mRNA in skeletal muscle and heart in response to sepsis. The gastrocnemius/plantaris complex or heart was removed from male mice 24 h after induction of peritonitis by cecal ligation and puncture (e.g., septic). Values are means ± SE; n = 9 each from two separate experiments. Data were normalized to L32 and the values for the control group set at 1.0 AU for each tissue. *P < 0.05, compared to control value from same tissue. Inset: representative Western blot of phosphorylation (P) at the Thr172 site of AMPKα, total AMPKα, phosphorylation at the Ser79 site of ACC, and total ACC in two skeletal muscles from control and septic mice. No change in AMPK or ACC phosphorylation was detected in cardiac muscle between control and septic mice (data not shown).

(bottom right panel), sepsis increased phosphorylation of both AMPKα (control = 1.00 ± 0.11 vs septic 2.23 ± 0.17 AU; P < 0.05; n = 9 per group) and ACC (1.00 ± 0.09 vs 1.78 ± 0.15 AU; P < 0.05; n = 9 per group). These sepsis-induced changes in phosphorylation were independent of a change in the total amount of either AMPKα or ACC. For cardiac muscle there was no difference in the phosphorylation of AMPKα and ACC detected between control and septic rats at the 24-h time point (data not shown).

Because sepsis activated AMPKα we used the pharmacologic agaent AICAR to directly activate AMPK [34]. Once taken up by intact cells AICAR is phosphorylated to form 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP), which is capable of producing stimulatory effects on AMPK identical to those of elevated AMP, but in the absence of detectable changes in adenine-nucleotide levels. For this study, mice were injected IP with AICAR and compared to appropriate time-matched saline-injected control animals at 6 h. This time point was selected based on data from preliminary studies indicating that IP injected AICAR increased the mRNA content of several FoxO mRNAs in skeletal muscle between 1-6 h. The maximal response occurred 3-6 h after addition of AICAR, but this increase was transient and not observed at either the 12 h or 24 h time point (data not shown).

In vivo administration of AICAR increased both AMPKα and ACC phosphorylation in skeletal muscle (**Figure 2**), but not in heart (data not

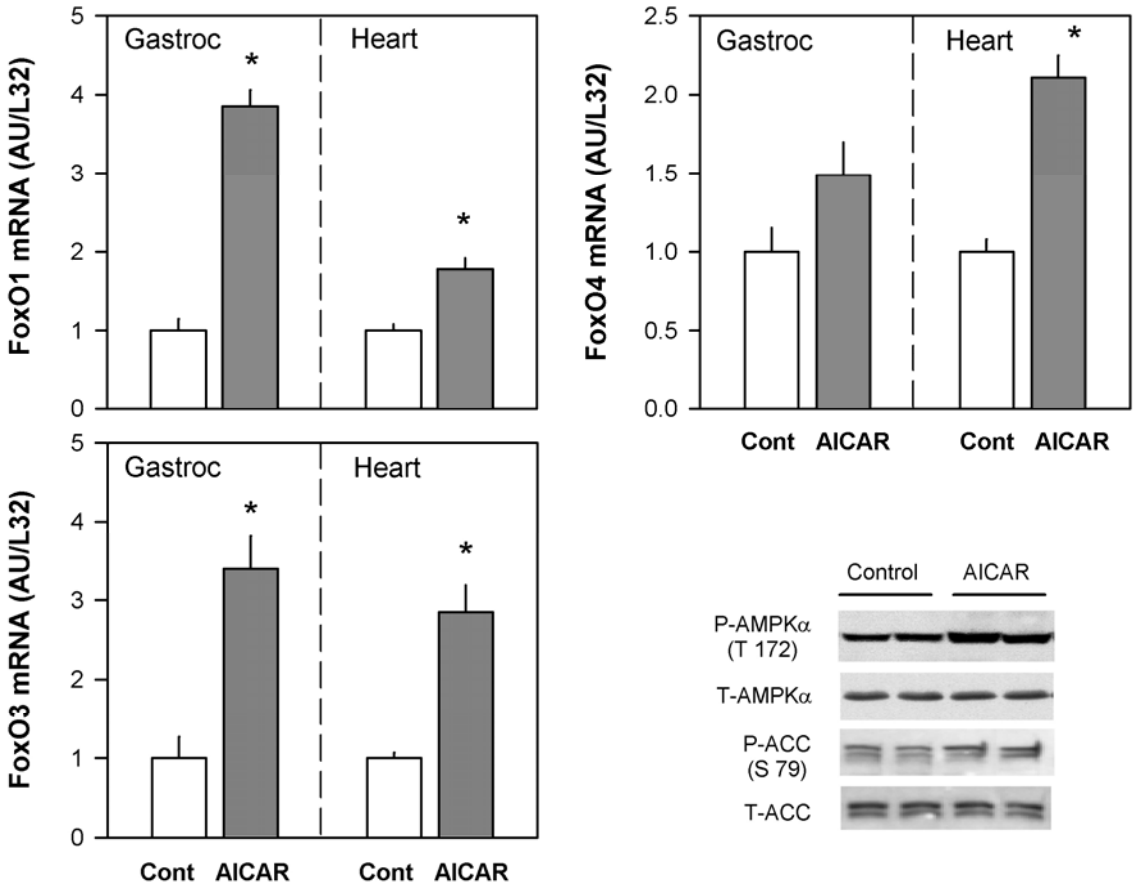

Figure 2. *In vivo* regulation of FoxO mRNA in skeletal muscle and heart in response to *in vivo* administered AICAR. The gastrocnemius/plantaris complex or heart was removed from male mice 6 h after in vivo administration of AICAR. Values are means ± SE; n = 5 and 7, respectively, from two separate experiments. Data were normalized to L32 and the values for the control group set at 1.0 AU for each tissue. *P < 0.05, compared to control value from same tissue. Inset: representative Western blot of phosphorylated (P) and total AMPKα and ACC in skeletal muscle from control and AICAR-treated mice. No change in AMPK or ACC phosphorylation was detected in cardiac muscle between control and AICAR-treated mice (data not shown).

shown). As with sepsis, AICAR selectively increased FoxO1 and FoxO3 mRNA in gastrocnemius (3- to 4-fold), but not FoxO4 mRNA (**Figure 2**). In contrast to the above mentioned results for sepsis, AICAR administration also increased FoxO1, -3, and -4 mRNA in cardiac muscle (1.7- , 2.8- and 2.0-fold, respectively; **Figure 2**).

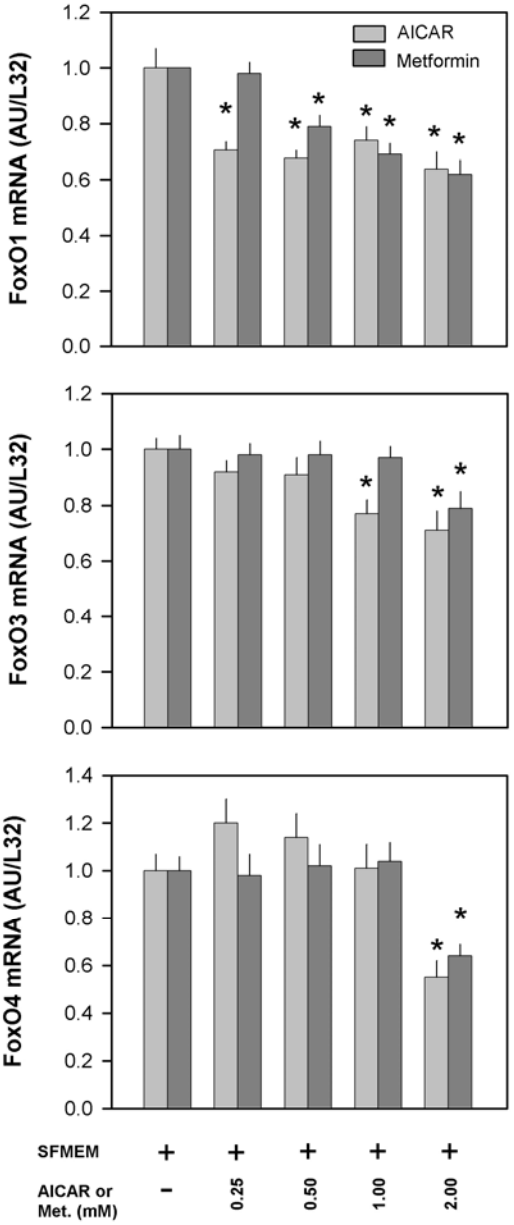

Figure 3. AICAR- and metformin-induced decreases in FoxO mRNA content. Bar graphs represent quantitation of FoxO1 (top panel), FoxO3 (middle panel), and FoxO4 (bottom panel) mRNA in C2C12 myotubes. Cells were incubated in the absence or presence of AICAR or metformin at the concentration indicated for 24 h. The value for AICAR-free control is set at 1.0 AU. Data were normalized to L32. Values represent mean ± SE for n = 8-9 per group. *P < 0.05, compared to control cells in the absence of AICAR or metformin.

In vitro AICAR and metformin decrease FoxO mRNA

To directly assess the sensitivity of FoxO mRNA to AMPK signaling, C2C12 myotubes were exposed to increasing concentrations of AICAR for 24 h (Figure 3). AICAR decreased the mRNA content for all three FoxOs with varying effectiveness. The AICAR-induced decrease in FoxO1 was first observed at a concentration of 0.25 mM, whereas higher doses of AICAR (1-2 mM) were required to consistently decrease mRNA content for FoxO3 and FoxO4. These AICAR-induced changes do not appear to result from a change in cell viability because LDH release into the culture media was not significantly increased at AICAR concentrations between 0.25-2 mM. When expressed as a percent of values from vehicle-treated time-matched control cells (100 ± 8%), LDH release averaged 110 ± 11%, 134 ± 18%, 124 ± 17%, 132 ± 11%, respectively, for the four AICAR concentrations used in Figure 3. In contrast, LDH release was markedly increased by myotubes treated with 5 mM AICAR (863 ± 127%; $P < 0.05$; $n = 8$). Additional time course studies were also performed using 2 mM AICAR. Results from these studies indicated that although AICAR tended to decrease FoxO-1, -3 and -4 mRNA at 8-16 h, only the reduction in mRNA content at the 24 h time point achieved statistical significance (data not shown). At no time point examined (i.e., 1, 2 , 4, 8, 16 or 24 h) did AICAR produce a statistically significant increase in the mRNA content for any of the three FoxOs, compared with time-matched control values (data not shown).

Conclusions regarding AMPK-dependent responses to AICAR are limited by potential non-AMPK "side effects" [34]. Therefore, myotubes were also treated with metformin to confirm the role of AMPK in modulating FoxO expression (**Figure 3**). Metformin is an anti-diabetic biguanide that activates AMPK in a mechanistically distinct manner from that of AICAR [35]. Similar to AICAR treatment, exposure of myotubes to increasing concentrations of metformin up to 2 mM decreased FoxO-1, -3 and -4 mRNA content (**Figure 3**). Again, this reduction in FoxO mRNA content was unlikely due to a change in cell

viability because LDH release by cells incubated with vehicle (100 ± 11%) and 2 mM metformin (127 ± 17% of control) for 24 h was not statistically different. FoxO1 appeared most sensitive to the effect of metformin which decreased mRNA content at a dose of 0.5 mM. The expression of FoxO3 and FoxO4 was only reduced when cells where incubated with 2 mM metformin. Additional time course studies were also performed using this metformin concentration. Results from these studies indicated that metformin significantly decreased FoxO-1, -3 and -4 mRNA at both 16 h and 24 h (data not shown). At no time point was the content for the three FoxO mRNAs statistically increased in metformin-treated cells when compared to values from time-matched control cells (data not shown).

Disruption of cellular energy homeostasis alters FoxO mRNA

While both AICAR and metformin are accepted pharmacological activators of AMPK, neither activates the kinase by directly disturbing the energy status of the cell. To determine if FoxO mRNA expression was sensitive to cellular energy starvation, C2C12 cells were incubated with 2-DG (**Figure 4**). 2-DG is a D-glucose analog which is phosphorylated but not further metabolized after cellular uptake. As a result, import of 2-DG inhibits hexokinase action through a negative feedback mechanism which ultimately restricts cellular glucose utilization [36]. Cells were incubated with 25 mM 2- DG for time periods up to 16 hours (beyond which some lethality resulted, unpublished observations). To account for any potential effect(s) of osmotic stress [an environmental stimulus itself capable of stimulating AMPK [35]] independent of energy depletion, equimolar amounts of D-mannitol were added to control cells. The presence of 25 mM mannitol did not appreciably shift the expression profile of any of the FoxO mRNAs during serum deprivation at the time point determined (data not shown).

Similar to the above-mentioned results with chemical activators of AMPK, the mRNA content of both FoxO1 and FoxO4 was decreased by 2-DG, especially between 8-16 h (**Figure 4**). In contrast, the mRNA content for FoxO3 was not significant reduced during the experimental protocol, and even demonstrated a transient increase of 25% at the 8 h time point (**Figure 4**).

To further confirm that authentic energy deprivation can regulate FoxO mRNA content,

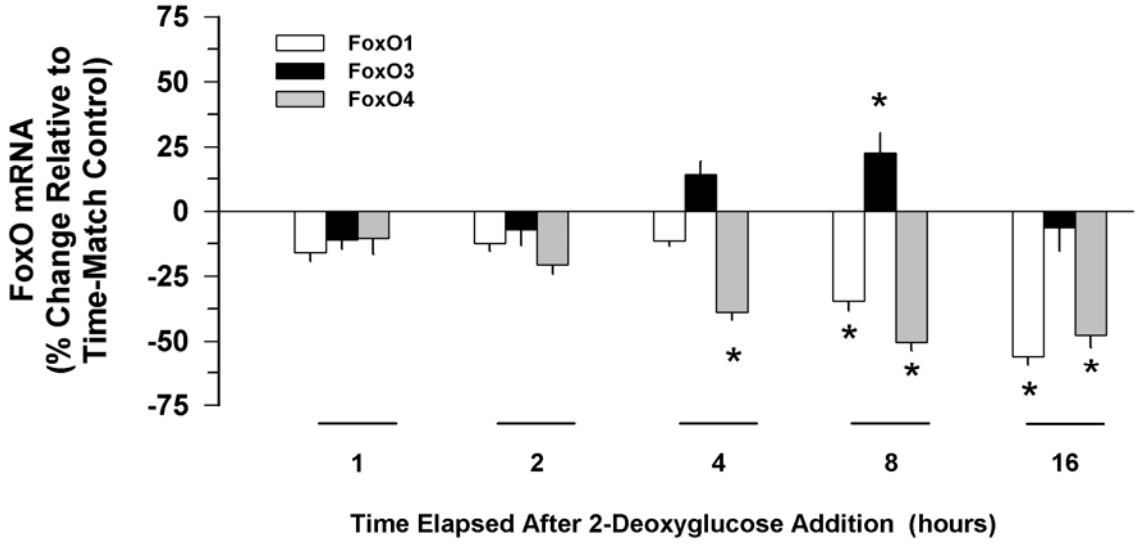

Figure 4. 2-Deoxyglucose (DG)-induced changes in FoxO mRNA content. Bar graph represents quantitation of FoxO1 (open bars), FoxO3 (black bars), and FoxO4 (light gray bars) mRNA in C2C12 myotubes. Cells were incubated for various times (1-16 h) after addition of 25 mM 2-DG. Time-matched control myotubes were incubated with 25 mM mannitol. The value for 2-DG-free control was set at 1.0 AU. Data were normalized to L32. Values represent mean ± SE for n = 8-9 per group. *P < 0.05, compared to control cells in the absence of 2-DG.

cells were incubated in SFMEM at various glucose concentrations. As illustrated in **Figure 5**, FoxO1 and FoxO4 mRNA was dose-dependently decreased when the media glucose was lowered from 5 mM (e.g., physiological) to 0 mM for 24 h. In contrast, while the expression of FoxO3 tended to be reduced by glucose deprivation, the changes failed to achieve statistical difference. Increasing the media glucose concentration from 5 mM to 25 mM for 24 h did not alter the mRNA content of any of the three FoxO mRNAs.

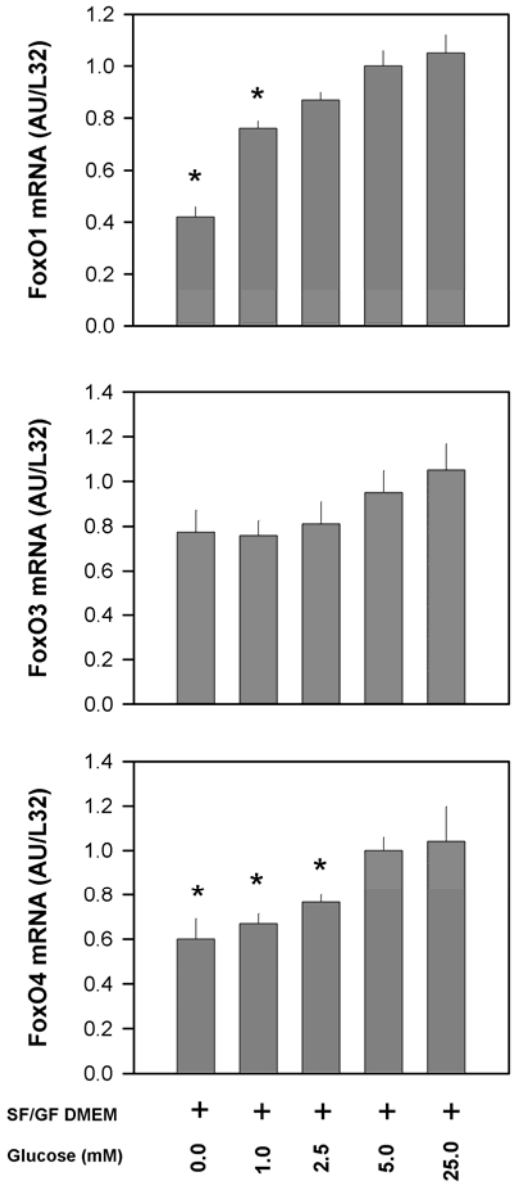

Figure 5. Alterations in FoxO mRNA content produced by changes in glucose concentration. Bar graphs represent quantitation of FoxO1 (top panel), FoxO3 (middle panel), and FoxO4 (bottom panel) mRNA in C_2C_{12} myotubes. Cells were incubated with the glucose concentration indicated for 24 h. The value for cells incubated with 5 mM glucose was set at 1.0 AU. Data were normalized to L32. Values represent mean ± SE for n = 8-9 per group. *P < 0.05, compared to cells incubated with 5 mM glucose which is considered physiological.

Activation of AMPK

To confirm AICAR, metformin and 2-DG activated AMPK, we assessed the phosphorylation of AMPK and ACC. Although representative Western blot data are presented in **Figure 6**, statistical analysis indicated AMPK phosphorylation was statistically increased ($P < 0.05$, n = 8-9 per group) by each AMPK activator (control = 100 ± 7 AU vs AICAR = 443 ± 58 AU; control = 100 ± 5 AU vs metformin 287 ± 36 AU; and control = 100 ± 9 AU vs 2-DG = 251 ± 22). These changes were independent of a change in the total amount of AMPK. Likewise, AICAR, metformin and 2-DG all increased ACC phosphorylation (2.4- to 4.9- fold) which was independent of a change in total ACC (**Figure 6**).

AMPK inhibition prevents AICAR-induced changes in myocyte protein balance

To address the specificity of the treatments used previously to activate AMPK, cells were cotreated with AICAR and compound C (**Figure 7**, top panel), a small molecule reversible inhibitor of AMPK [37]. Initial dose-response experiments determined that a concentration of 20μM was optimal [23]. As illustrated in Figure 7, AICAR alone decreased protein synthesis 30-50% and increased protein degradation 15-20%. A similar effect was seen in response to metformin (data not shown). Preincubation of myotubes with the AMPK inhibitor compound C prevented the AICAR-induced increase in protein synthesis and decrease in protein degradation. Furthermore, compound C prevented the AICAR-induced decreased in FoxO-1, -3 and -4 mRNA content in C2C12 myotubes (**Figure 7**, bottom panel).

Discussion

The FoxO transcription factors regulate a diverse array of physiological processes and have been functionally linked to changes in protein balance [12,13,38]. The overwhelming majority of the data pertaining to the FoxOs relates to their posttranslational control by

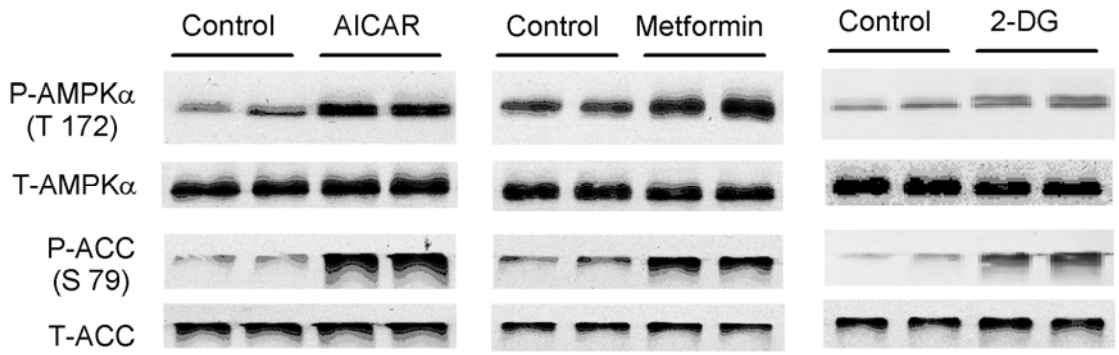

Figure 6. Phosphorylation of AMPK and ACC in response to AICAR, metformin and 2-DG. Representative immunoblots of phosphorylation at the Thr172 site of AMPKα, total AMPKα, phosphorylation at the Ser79 site of ACC, and total ACC. Myotubes were incubated with either AICAR (2 mM for 24 h), metformin (2 mM for 24 h), or 2-DG (25 mM for 16 h), and then cells isolated for Western blot analysis. Blots are representative of a sample size equal to 8-9 per group. There was no change in either the total amount of AMPK or ACC (Figure 6) or β-tubulin (data not shown) for any of the three AMPK activators.

phosphorylation, acetylation or ubiquitination [4], whereas other levels of FoxO regulation (e.g., mRNA expression and tissue specificity) have been less well studied. To address this gap in understanding, the stated aim of the present investigation was to determine how various metabolic stresses, such as those that might be seen during infection and inflammation, alter the expression of the principle FoxO mRNAs in striated muscle.

Many of the canonical growth-controlling pathways promoting protein accretion are also involved in suppressing the presentation of classic markers of wasting muscle and/or the atrophic process itself. The importance of AMPK as a central regulator of cellular metabolism during times of limited energy availability is well appreciated (14). In several of these contexts (e.g., nutritional starvation and sepsis) there is both an acceleration of proteolysis and inhibition of protein synthesis in skeletal muscle which, when prolonged, leads to debilitating wasting. Likewise, AMPK activation also impacts protein balance by its reciprocal regulation of these processes. For example, AICAR has been shown to decrease muscle protein synthesis under both in vivo [20] and in vitro [21,22] conditions. Furthermore, AICAR also increases protein degradation, at least under in vitro conditions [24 and current data], and this finding is consistent with the marked increase in the expression of the atrogenes atrogin-1 and MuRF1-known regulators of ubiquitin-mediated proteolytic activity [39] - under both in vivo and in vitro conditions [23]. Our current data represent the initial report of sepsis increasing AMPK phosphorylation in skeletal, but not cardiac, muscle.

This sepsis-induced increase in AMPK activation was associated with the increased expression of FoxO1 and FoxO3 in the gastrocnemius. In contrast, sepsis did not altered skeletal muscle FoxO4 mRNA content at least at the two time points (6 h and 24 h) assessed. These data are consistent with previous in vivo studies reporting that exercise, diabetes, uremia and cancer also increase FoxO1 and Foxo3 mRNA in skeletal muscle, with little or no change in FoxO4 (6,8-10). Together these data imply the physiological role for FoxO4 differs from that of the other FoxOs implying these FoxO family members have distinct and nonoverlapping functions in skeletal muscle, as proposed in other tissues [40]. Furthermore, sepsis did not alter FoxO expression in heart at either 6 h or 24 h, suggesting that changes in FoxO mRNA content may be more pertinent to metabolic regulation in skeletal muscle than in heart. Our data also indicate a qualitatively comparable increase in FoxO1 and FoxO3 mRNA in skeletal muscle when AICAR is used in vivo to activate AMPK. However, in contrast to sepsis, AICAR also significantly increased the mRNA content for all three FoxO mRNA transcripts in heart without altering AMPK or ACC phosphorylation. The reason for this apparent difference in the

response of skeletal and cardiac muscle to in vivo administered AICAR is unknown. Because only two time points were assessed under in vivo conditions, we cannot exclude the possibility that AMPK activation/deactivation in heart was more transient than in skeletal muscle. Alternatively, the in vivo injection of AICAR might be expected to lead to the generation of a number of secondary mediators which, independent of AMPK activation, may increase FoxO mRNA content in both heart and skeletal muscle.

To directly evaluate the mechanism by which AMPK activation increases FoxO mRNA in muscle, additional studies were performed using C2C12 myotubes. Herein the role of

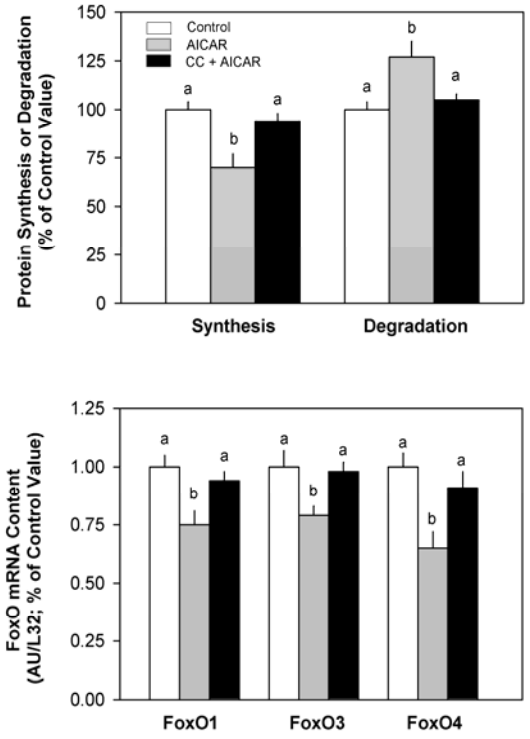

Figure 7. AICAR-induced changes in protein synthesis and degradation. Top panel, the in vitro rate of protein synthesis and degradation was determined as described in METHODS in C_2C_{12} myotubes incubated with either vehicle (control), AICAR or coincubation of compound C (CC) + AICAR. Values are means ± SE, n = 7-9 per group. Bottom panel, represents quantitation of FoxO1, FoxO3, and FoxO4 mRNA in C_2C_{12} myotubes at various time points after addition of vehicle (control), AICAR or coincubation of CC with AICAR. The value for the vehicle control was set at 1.0 AU. Data were normalized to L32. Values represent mean ± SE for n = 8-9 per group. For both bar graphs, values with different small case letters are significantly different ($P < 0.05$) from each other.

AMPK in regulating FoxO expression was demonstrated using three independent agents capable of activating AMPK in distinctive manners – AICAR, metformin, and glucose depletion. While AICAR and 2-DG stimulate the kinase by acting as an AMP-mimetic or placing cells directly under energetic duress (respectively), metformin stimulates the kinase without detectably perturbing AMP or ATP content [35,41]. The biguanide appears to function by inhibiting complex I of the respiratory chain and the increased production of mitochondrial-derived reactive nitrogen species [35,42]. In general, treatment of myotubes with these AMPK activators decreased the FoxO mRNAs. FoxO1 and FoxO4 were all decreased by AICAR and metformin as well as by glucose deprivation induced by either 2-DG or lowering of the media glucose concentration. In addition, AICAR and metformin also decreased FoxO3 mRNA content. For each stimuli which increased AMPK phosphorylation, the decreased expression of FoxO1 appeared quantitatively greatest. While none of these reagents is entirely specific in stimulating AMPK, that similar results were achieved using each suggests the changes in FoxO gene expression may be AMPK-dependent and not a function of some alternative kinase or signaling pathway. Our ability to essentially prevent the AICAR-induced decrease in FoxO mRNAs in myotubes incubated with compound C supports such a direct mechanism. However, it is noteworthy that in general the AICAR-induced change in FoxO mRNA in myotubes was qualitatively different from the response observed in vivo (e.g., decrease versus increase, respectively). We do not believe this difference is related to the use of the C2C12 myocytes per se because other studies in our laboratory have indicated a comparable decrease in FoxO-1, -3 and -4 mRNA in L6 myocytes (Lang, unpublished data). Furthermore, these changes are unlikely to result from a generalized "toxic effect" of the AMPK activators because the doses of AICAR and metformin used in the current study do not appear to alter cell viability, as evidenced by the lack of an increase in LDH release. Finally, because our time course studies with AICAR and metformin in C2C12 myotubes did not reveal a statistically significant increase in any FoxO mRNA in response to either agent, we do not believe the difference in the timing of the in vivo and in vitro studies provides a likely explanation. However, a possibility explanation

for the differential response between myocytes in vitro and muscle in vivo is the former experimental condition represents a direct effect of AMPK activation, while the latter represents the composite metabolic and gene response to both direct AMPK activation as well as the indirect effect of other hormonal and circulating mediators.

Although the physiological consequences of these changes in FoxO mRNA were not directly assessed in the current study, we did note an association with the decrease in FoxOs, the activation of AMPK, and protein balance in incubated myotubes. In this regard, both AICAR and metformin decreased protein synthesis and increased protein degradation in C2C12 myotubes. Importantly, treatment with the AMPK antagonist compound C prevented these AICAR-induced changes in protein metabolism, both synthesis and degradation. These data are consistent with the previously reported ability of in vivo-administered AICAR to decrease protein synthesis and to increase the expression of the atrogenes atrogin-1 and MuRF1 in skeletal muscle [20,23]. Although compound C also prevented the AICAR-induced decrease in FoxO mRNA in myotubes, collectively our data do not support a causative role for a change in FoxO mRNAs in regulating protein balance. For example, under in vivo conditions FoxO1 and FoxO4 mRNAs are increased by AICAR (and sepsis) whereas AICAR decreases FoxO1, -3 and -4 under in vitro conditions; however, we would anticipate that muscle protein synthesis is decreased and protein degradation increased under both in vivo and in vitro conditions [20,23,43].

In conclusion, we describe herein the role of AMPK in controlling the mRNA content of FoxO family mRNAs using an in vitro model of skeletal muscle. Exposure of differentiated C2C12 cells to agonists of AMPK generally produced a dose-dependent decrease in the mRNA content for FoxO1, FoxO3 and FoxO4. Moreover, chemical inhibition of AMPK prevented both the AICAR-induced decrease in FoxO mRNAs as well as the concomitant alterations in protein metabolism. Surprisingly, for the most part, the directionality of the changes produced by in vitro activation of AMPK did not mimic the changes produced in vivo by either AICAR stimulation or inflammation produced by sepsis despite protein synthesis being reduced and protein degradation being increased under both conditions. Together these data suggest muscle FoxO mRNA content may be regulated in vivo by other hormonal or nutrient signals acting independently of AMPK activation and that such changes in FoxO mRNAs can be dissociated from changes in protein metabolism.

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References

[1] Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell 2004; 117: 421-426.

[2] Anderson MJ, Viars CS, Czekay S, Cavenee WK, Arden KC. Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. Genomics 1998; 47: 187-199.

[3] Barthel A, Schmoll D, Unterman TG. FoxO proteins in insulin action and metabolism. Trends Endocrinol Metab 2005; 16: 183-189.

[4] van der Heide LP, Hoekman MF, Smidt MP. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J 2004; 380: 297- 309.

[5] Plas DR, Thompson CB. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. J Biol Chem 2003; 278: 12361-12366.

[6] Furuyama T, Yamashita H, Kitayama K, Higami Y, Shimokawa I, Mori N. Effects of aging and caloric restriction on the gene expression of Foxo1, 3, and 4 (FKHR, FKHRL1, and AFX) in the rat skeletal muscles. Microsc Res Tech 2002; 59: 331-334.

[7] Hosaka T, Biggs WH, III, Tieu D, Boyer AD, Varki NM, Cavenee WK, Arden KC. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A 2004; 101: 2975-2980.

[8] Kamei Y, Mizukami J, Miura S, Suzuki M, Takahashi N, Kawada T, Taniguchi T, Ezaki O. A forkhead transcription factor FKHR up-

regulates lipoprotein lipase expression in skeletal muscle. FEBS Lett 2003; 536: 232-236.
[9] Jorgensen SB, Wojtaszewski JF, Viollet B, Andreelli F, Birk JB, Hellsten Y, Schjerling P, Vaulont S, Neufer PD, Richter EA, Pilegaard H. Effects of alpha-AMPK knockout on exerciseinduced gene activation in mouse skeletal muscle. FASEB J 2005; 19: 1146-1148.
[10] Lecker SH, Jagoe RT, Gilbert A, Gomes M, Baracos V, Bailey J, Price SR, Mitch WE, Goldberg AL. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. FASEB J 2004; 18: 39-51.
[11] Imae M, Inoue Y, Fu Z, Kato H, Noguchi T. Gene expression of the three members of hepatocyte nuclear factor-3 is differentially regulated by nutritional and hormonal factors. J Endocrinol 2000; 167: R1-R5.
[12] Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 2004; 117: 399-412.
[13] Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 2004; 14: 395-403.
[14] Hardie DG. The AMP-activated protein kinase pathway--new players upstream and downstream. J Cell Sci 2004; 117: 5479-5487.
[15] Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, Yang Q, Bennett C, Harada Y, Stankunas K, Wang CY, He X, MacDougald OA, You M, Williams BO, Guan KL. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell 2006; 126: 955-968.
[16] Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003; 115: 577-590.
[17] Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ, Thompson CB. AMPactivated protein kinase induces a p53-dependent metabolic checkpoint. Mol Cell 2005; 18: 283-293.
[18] Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 2005; 1: 15-25.
[19] Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest 2006; 116: 1776-1783.
[20] Bolster DR, Crozier SJ, Kimball SR, Jefferson LS. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. J Biol Chem 2002; 277: 23977-23980.
[21] Du M, Shen QW, Zhu MJ, Ford SP. Leucine stimulates mammalian target of rapamycin signaling in C2C12 myoblasts in part through inhibition of adenosine monophosphate-activated protein kinase. J Anim Sci 2007; 85: 919-927.
[22] Williamson DL, Bolster DR, Kimball SR, Jefferson LS. Time course changes in signaling pathways and protein synthesis in C2C12 myotubes following AMPK activation by AICAR. Am J Physiol Endocrinol Metab 2006; 291: E80-E89.
[23] Krawiec BJ, Nystrom GJ, Frost RA, Jefferson LS, Lang CH. AMP-activated protein kinase agonists increase mRNA content of the muscle-specific ubiquitin ligases MAFbx and MuRF1 in C2C12 cells. Am J Physiol Endocrinol Metab 2007; 292: E1555-E1567.
[24] Nakashima K, Yakabe Y. AMPK activation stimulates myofibrillar protein degradation and expression of atrophy-related ubiquitin ligases by increasing FOXO transcription factors in C2C12 myotubes. Biosci Biotechnol Biochem 2007; 71: 1650-1656.
[25] Berasi SP, Huard C, Li D, Shih HH, Sun Y, Zhong W, Paulsen JE, Brown EL, Gimeno RE, Martinez RV. Inhibition of gluconeogenesis through transcriptional activation of EGR1 and DUSP4 by AMP-activated kinase. J Biol Chem 2006; 281: 27167-27177.
[26] Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M, Cantley LC. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 2005; 310: 1642-1646.
[27] Woods A, zzout-Marniche D, Foretz M, Stein SC, Lemarchand P, Ferre P, Foufelle F, Carling D. Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. Mol Cell Biol 2000; 20: 6704-6711.
[28] Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, Young LH, Semenkovich CF, Shulman GI. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. Am J Physiol Endocrinol Metab 2001; 281: E1340-E1346.
[29] Nilsson EC, Long YC, Martinsson S, Glund S, Garcia-Roves P, Svensson LT, Andersson L, Zierath JR, Mahlapuu M. Opposite transcriptional regulation in skeletal muscle of AMPactivated protein kinase gamma3 R225Q transgenic versus knock-out mice. J Biol Chem 2006; 281: 7244-7252.
[30] Holmes BF, Sparling DP, Olson AL, Winder WW, Dohm GL. Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase. Am J Physiol Endocrinol Metab 2005; 289: E1071-E1076.
[31] Lang CH, Frost RA. Glucocorticoids and

TNFalpha interact cooperatively to mediate sepsis-induced leucine resistance in skeletal muscle. Mol Med 2006; 12: 291-299.
[32] Giegerich R, Meyer F, Schleiermacher C. GeneFisher--software support for the detection of postulated genes. Proc Int Conf Intell Syst Mol Biol 1996; 4: 68-77.
[33] Stein SC, Woods A, Jones NA, Davison MD, Carling D. The regulation of AMP-activated protein kinase by phosphorylation. Biochem J 2000; 345 Pt 3: 437-443.
[34] Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem 1995; 229: 558-565.
[35] Fryer LG, Parbu-Patel A, Carling D. The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. J Biol Chem 2002; 277: 25226-25232.
[36] Geschwind JF, Georgiades CS, Ko YH, Pedersen PL. Recently elucidated energy catabolism pathways provide opportunities for novel treatments in hepatocellular carcinoma. Expert Rev Anticancer Ther 2004; 4: 449-457.
[37] Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001; 108: 1167-1174.
[38] Allen DL, Unterman TG. Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors. Am J Physiol Cell Physiol 2007; 292: C188-C199.
[39] Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 2001; 294: 1704-1708.
[40] Kajihara T, Jones M, Fusi L, Takano M, Feroze-Zaidi F, Pirianov G, Mehmet H, Ishihara O, Higham JM, Lam EW, Brosens JJ. Differential expression of FOXO1 and FOXO3a confers resistance to oxidative cell death upon endometrial decidualization. Mol Endocrinol 2006; 20: 2444-2455.
[41] Hawley SA, Gadalla AE, Olsen GS, Hardie DG. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. Diabetes 2002; 51: 2420-2425.
[42] Xie Z, Dong Y, Zhang M, Cui MZ, Cohen RA, Riek U, Neumann D, Schlattner U, Zou MH. Activation of protein kinase C zeta by peroxynitrite regulates LKB1-dependent AMP-activated protein kinase in cultured endothelial cells. J Biol Chem 2006; 281: 6366-6375.
[43] Lang CH, Frost RA, Vary TC. Regulation of muscle protein synthesis during sepsis and inflammation. Am J Physiol Endocrinol Metab 2007; 293: E453-E459.