Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase

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Holmes, Burton F., David P. Sparling, Ann Louise Olson, William W. Winder, and G. Lynis Dohm. Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase. Am J Physiol Endocrinol Metab 289:E1071–E1076, 2005. First published August 16, 2005; doi:10.1152/ajpendo.00606.2004.—As the primary glucose transporter in skeletal muscle, GLUT4 is an important factor in the regulation of blood glucose. We previously reported that stimulation of AMP-activated protein kinase (AMPK) with 5-aminomidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) increased GLUT4 expression in muscle. GLUT4 enhancer factor (GEF) and myocyte enhancer factor 2 (MEF2) have been shown to be important for normal GLUT4 expression because deletion or truncation of the consensus sequences on the promoter causes depressed GLUT4 mRNA expression.

This led to the current study to investigate possible roles for GEF and MEF2 in mediating the activation of GLUT4 gene transcription in response to AMPK. Here we show that, although AMPK does not appear to be directly involved in the phosphorylation of MEF2A, AMPK directly phosphorylates the GEF protein in vitro. MEF2 and GEF are activated in response to AMPK as we observed translocation of both to the nucleus after AICAR treatment. Nuclear MEF2 protein content was increased after 2 h, and GEF protein was increased in the nucleus 1 and 2 h post-AICAR treatment. Last, GEF and MEF2 in binding to the GLUT4 promoter within 2 h after AICAR treatment. Thus we conclude that GEF and MEF2 mediate the AMPK-induced increase in transcription of skeletal muscle GLUT4. AMPK can phosphorylate GEF and in response to AICAR, GEF, and MEF2 translocate to the nucleus and have increased binding to the GLUT4 promoter.

5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside; glutathione S-transferase; glucose transporter 4

AS THE PRIMARY GLUCOSE TRANSPORTER in skeletal muscle, GLUT4 is an important factor in the regulation of blood glucose (3, 6, 17). Previous research from our laboratory has shown an increase in skeletal muscle GLUT4 mRNA expression and protein levels due to activation of AMP-activated protein kinase (AMPK) after 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment (23). Understanding the interactions between GLUT4 and AMPK is important in the effort to treat glucose insensitivity present in the obese and those suffering from diabetes. Olson and collaborators (10, 15, 19, 23) have shown that GLUT4 enhancer factor (GEF) and myocyte enhancer factor 2 (MEF2) are important transcription factors required for normal GLUT4 expression, which led to our current study to determine the possible role of GEF and MEF2 in mediating the activation of GLUT4 gene transcription in response to AMPK activation.

MEF2 is a transcription factor with many functions, including embryonic development in the brain, heart, and skeletal muscle (13). It is also regulated by various factors, such as Ca2+ levels in the muscle and dephosphorylation by calcineurin (21). MEF2 contains a known nuclear localization sequence encompassing amino acids 472–507 in the primary sequence (22), indicating that MEF2 can be activated to translocate to the nucleus. More recent work indicates that MEF2 colocalizes to the nucleus with histone deacetylase 4 (1).

The MEF2 isoforms active in skeletal muscle are A, C, and D (11, 19), and, in fully developed skeletal muscle, MEF2 is necessary to increase GLUT4 mRNA expression (18, 19). In mobility shift assays, Thai et al. (19) have shown that, in vitro, translated MEF2A and MEF2C have the ability to carry out specific binding to the GLUT4 MEF2 binding sequence. Using constructs with the GLUT4 promoter and luciferase reporter, gene-specific regions were identified as necessary for GLUT4 expression. One of these regions, from −522 to −420 bp of the human GLUT4 promoter, contains an E-box and an MEF2 binding site (2). Mutations of the MEF2 binding site resulted in a nearly complete loss of reporter gene expression (2). Likewise, a mutation of the MEF2 binding site in the GLUT4 promoter region in C2C12 cells results in a loss of function and decreased GLUT4 mRNA expression (8). The results of these studies indicate that, while necessary, MEF2 is not sufficient for normal GLUT4 expression (19).

In a follow up to their previous work, Oshel et al. (15) identified a 30-bp regulatory element they named Domain I, located upstream from the GLUT4 promoter (−742 to −712 bp upstream from the initiation site of the human GLUT4 promoter). They cloned the protein that binds to Domain I and named it GEF (15). GEF consists of an 1,100-bp gene that encodes a 50-kDa peptide. GEF binds specifically to Domain I and could be competed by an unlabeled wild-type oligonucleotide. The CDNA sequence also contains a nuclear localization sequence indicating that it can be localized to the nucleus. Deletion or mutation of the Domain I binding site results in a decrease in gene expression or inhibits complex formation, respectively (15). This indicates that the Domain I regulatory element and the GEF protein are necessary for normal GLUT4 mRNA expression. Therefore, both MEF2 and GEF protein binding are necessary for normal GLUT4 mRNA expression (15).
AMPK REGULATION OF GEF AND MEF2

Our current studies focus on the role these transcription factors play in regulation of GLUT4 transcription. We hypothesize that AMPK regulates GLUT4 mRNA expression, either directly or through cofactors, by acting on MEF2 and GEF to cause them to move to the nucleus and bind to the GLUT4 promoter. Herein, we describe some of the direct effects of AMPK activation on MEF2 and GEF activity. Specifically, we measured phosphorylation of GEF and MEF2A, translocation to the nucleus, and DNA binding activity of MEF2 and GEF to the GLUT4 promoter.

METHODS

This study consisted of two experiments. In the first experiment, the phosphorylation of recombinant MEF2A and GEF by purified AMPK was investigated. In the second experiment, rats were injected with AICAR, and animals were killed 1, 2, 5, and 12 h later. Muscle was harvested, nuclei were isolated, and the protein content of MEF2 and GEF, as well as their binding to DNA sequences of the human GLUT4 promoter, were determined.

Animal care and housing. All procedures were approved by the Institutional Animal Care and Use Committee of East Carolina University. Animals were injected with AICAR (0.5 mg/g body wt; Toronto Research Chemicals, North York, ON, Canada), and the gastrocnemius plantaris muscle group was removed at 1, 2, 5, and 12 h postinjection. Control groups were injected with saline (0.9% sterile saline, 0.1 ml/100 g body wt).

Phosphorylation assays. The phosphorylation procedure was a modification of the protocol described by Winder and Hardie (20). Briefly, recombinant GEF and MEF2A were incubated with purified AMPK and radioactive ATP (20), and phosphorylation status was measured. Acetyl-CoA carboxylase (ACC) isolated from rat liver was used as a positive control for AMPK activity and phosphorylation. Samples were then loaded on a 4–20% Tris/HCl ready gel (Bio-Rad), proteins were transferred to a polyvinylidene difluoride membrane at 100 V for 120 min. The membranes were blocked with 5% milk Tris-buffered saline-Tween 20 (TBS-T) for 30 min. Membranes were incubated overnight with primary antibody in 5% milk in TBS-T. Primary antibody for MEF2 and ACC was purchased from Santa Cruz (Beverly, CA), and primary antibody for the GST-GEF fusion protein was isolated and purified from rabbit serum by protein-G affinity chromatography (15). After four 5-min washes in TBS-T, membranes were exposed to horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) at 1/10,000 dilution in TBS-T. Western immunoblot. Muscle samples and nuclei isolated from rats were homogenized with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment. Rats were AICAR injected and killed at various times. The gastrocnemius-planta
tis muscle group was homogenized, and GLUT4 values were measured by real-time quantitative PCR (RTQ-PCR) for GLUT4 mRNA and graphed relative to control; n = 5 animals for all groups. *P = 0.026 and †P = 0.015 vs. control.

Fig. 1. Phosphorylation of GLUT4 enhancer factor (GEF) by AMP-activated protein kinase (AMPK). Liver acetyl-CoA carboxylase (ACC) and recombinant GEF and myocyte enhancer factor (MEF2)A were incubated under the following 3 conditions: control, AMPK, and AMPK + AMP. These gels are representative of 3 experiments.

Fig. 2. Time course for upregulation of GLUT4 mRNA in rat skeletal muscle with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment. Rats were AICAR injected and killed at various times. The gastrocnemius-planta
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Table 1. Time course of upregulation of GLUT4 mRNA in rat skeletal muscle with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment. Rats were AICAR injected and killed at various times. The gastrocnemius-planta
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Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSA) were performed as described previously (15). Briefly, oligonucleotides containing the Domain I, GEF binding site (CTT-GTCCCTCGGACCGGCTCCAGGAACCAA), and the complementary strand were custom synthesized (Sigma) and end labeled with T4 polynucleotide kinase. Dried gels were placed on a PhosphorImager (Molecular Dynamics/Amersham) overnight, and quantification was made using Gel-Pro Analyzer 4.0 (Media Cybernetics).

DNA-binding assay. Nuclear extracts from the AICAR-treated rat gastrocnemius were used to determine changes in MEF2 binding to the DNA binding site using the TransAM DNA-binding assay for MEF2 (Active Motif, Carlsbad, CA). After nuclear isolation, as described above, the samples were treated as outlined in the Active Motif protocol.

Statistical analysis. Mean differences from each experiment were analyzed by ANOVA, applying Tukey’s post hoc test when significance was found. Statistical significance was set at \( P \leq 0.05 \).

RESULTS

The first objective of this study was to determine if AMPK can directly phosphorylate MEF2A and GEF. As a positive control, we used ACC phosphorylation by AMPK (Fig. 1). MEF2A was not directly phosphorylated by in vitro treatment with AMPK or AMPK + AMP (Fig. 1A). However, using the same in vitro phosphorylation assays, we have found that AMPK directly phosphorylates GEF in the presence of AMPK, AMP, and [\( \gamma^{-32}P \)]ATP (Fig. 1). GST, the fusion protein used for GEF isolation, was also examined for in vitro phosphorylation and was not phosphorylated (unpublished data). Western blots show that the phosphorylation is not the result of increased protein in the lane (not shown).

We previously reported that AICAR treatment increased muscle GLUT4 protein and mRNA (23). In this study, we confirm that, 5 and 12 h after AICAR treatment, GLUT4 mRNA is elevated above control (Fig. 2). As an indication of AMPK activity, we measured phospho-ACC levels in whole muscle homogenate and found phospho-ACC increases two-fold at 1 h post-AICAR treatment (Fig. 3A). Because AMPK can be translocated to the nucleus, nuclear phospho-AMPK (pAMPK) protein was measured by Western blot analysis and found to be significantly increased with AICAR treatment compared with saline-treated controls. Nuclear phospho-AMPK was increased at 5 h post-AICAR treatment compared with saline-treated controls (Fig. 3B).

Nuclear GEF protein levels were measured by Western blot as an indication of nuclear translocation. Nuclear GEF was significantly increased 1 h (\( P = 0.024 \)) and 2 h (\( P = 0.032 \)) post-AICAR treatment compared with saline-treated controls (Fig. 4; \( n = 6 \) for all groups). There is no change in whole muscle GEF protein levels (not shown). To investigate the possible activation of MEF2 by AMPK, we measured changes

Fig. 3. A: total phospho-ACC (pACC) protein levels. Rats were AICAR injected and killed at various times. The gastrocnemius-plantaris muscle group was homogenized, and pACC was determined by Western blot; \( n = 6 \) for all groups. *\( P = 0.04 \). B: phospho-AMPK (pAMPK) translocation to the nucleus. Rats were AICAR injected and killed at various times. The gastrocnemius-plantaris muscle group was homogenized, and pAMPK was determined by Western blot; \( n = 6 \) for all groups. *\( P = 0.01 \).

Fig. 4. GEF protein in nuclei and whole homogenate. Rats were AICAR or saline injected and killed at various times, and nuclei were isolated for Western blot analysis of GEF. *\( P = 0.024 \) and †\( P = 0.032 \).
Because transcriptional activation of GLUT4 by GEF and MEF2 involves binding to DNA and translocation to the nucleus, binding of these proteins to the consensus sequences of the human GLUT4 promoter was investigated. Changes in GEF binding to the Domain I consensus sequence of human GLUT4 promoter was measured by EMSA. A twofold increase in GEF binding was seen at 1 h ($P = 0.007$) and 2 h ($P = 0.018$) post-AICAR treatment but not at 5 h posttreatment ($n = 6$ for all groups; Fig. 6).

MEF2 binding at the MEF2 consensus sequence was measured using the TransAM DNA binding kit. MEF2 binding to DNA was increased twofold ($P = 0.005$) at 2 h post-AICAR treatment. There was no significant difference at 1 and 5 h posttreatment (Fig. 7; $n = 6$ for all groups).

**DISCUSSION**

The purpose of the current study was to better understand the links between AMPK activation and regulation of GLUT4 expression through activation of the transcription factors GEF and MEF2. We previously reported that GLUT4 mRNA increases with exercise and AICAR treatment and that GLUT4 protein increases after 5 days of AICAR treatment or 7–10 days of exercise in humans (4, 5, 9, 10, 23). MEF2 and GEF were implicated in the regulation of GLUT4 mRNA expression because the truncation or deletion of the binding sequence for either of these transcription factors on the GLUT4 promoter

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**Fig. 5.** MEF2 protein in nuclei and whole homogenate. Animals were injected with 0.5 mg/g body wt AICAR, and gastrocnemius was taken at 1, 2, and 5 h postinjection. Controls were saline injected with an equal volume of 0.9% saline solution. A: MEF2 protein translocation to the nucleus in rat skeletal muscle treated with AICAR and measured at various time points. MEF2 in the nucleus is significantly increased over control values at 2 h posttreatment. *$P = 0.036$. B: MEF2 protein in whole muscle homogenates at times post-AICAR treatment; $n = 6$ for all groups. *$P = 0.011$. 

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**Fig. 6.** GEF binding to DNA after AICAR treatment measured by EMSA in rat skeletal muscle. A: time course for GEF binding to DNA. A 2-fold increase was seen at 1 h ($P = 0.007$) and 2 h ($P = 0.018$) post-AICAR treatment. B: representative blot of control and 1 h, with increasing concentrations of cold competitor ($n = 6$ for all groups).
region decreases GLUT4 mRNA expression (15, 19), and the exercise or AICAR responses are lost when the GEF or MEF2 promoter binding sites are altered (10, 23). Because AMPK has been implicated as being important in the regulation of GLUT4 mRNA expression, this study was designed to better understand the regulation of GEF and MEF2 by AMPK.

There are at least three ways that transcription factors can be regulated. First, the transcription factor can be phosphorylated, causing it to translocate to the nucleus, where it binds to the appropriate promoter to initiate transcription. The possibility of phosphorylation in the nucleus and increased DNA binding also exists. Second, increased expression of a transcription factor also increases the probability of nuclear translocation with subsequent regulation of the target gene. Third, binding of the transcription factor with coactivators to increase translocation to the nucleus and/or increase binding to the target promoter to enhance gene expression has been demonstrated. We have endeavored to investigate these three possibilities in the regulation of GEF and MEF2 by AMPK.

Are GEF and MEF2 substrates for AMPK? This was the first question that we investigated. The results show that GEF is phosphorylated in vitro by AMPK with the addition of AMP. Nuclear GEF protein levels increase in skeletal muscle with AICAR treatment, suggesting GEF may be phosphorylated and translocate to the nucleus. GEF has a known nuclear localization sequence (15), making translocation to the nucleus after phosphorylation a possible mechanism for GEF regulation. The nuclear GEF content increases at 1 and 2 h posttreatment, and GEF binding increases as well. These data support the previous findings that GEF is necessary for increased GLUT4 mRNA expression.

MEF2 was not directly phosphorylated in our in vitro system. This suggests it is not a direct substrate for AMPK, but we cannot rule out the possibility that MEF2 is indirectly phosphorylated by another kinase that is activated by AMPK. A second possibility exists that a coactivator is phosphorylated, increasing its binding to MEF2 with subsequent translocation to the nucleus.

The significant increase in total MEF2 protein is of interest. This indicates that AMPK activation could lead to increased MEF2 mRNA and protein content in skeletal muscle. The total muscle protein increases roughly twofold with AICAR treatment, and nuclear protein increased fivefold, indicating the increase in nuclear MEF2 is not simply because of increases in total protein. The twofold increase in MEF2 binding to the consensus sequence 2 h post-AICAR treatment fits with the timeline shown for translocation, and increased DNA binding to the promoter suggests MEF plays a role in GLUT4 mRNA expression.

### Fig. 7. MEF2 protein binding to MEF2 DNA binding site after AICAR treatment
DNA binding was measured in nuclear extracts by the TransAM Assay (ACTIV MOTIF). At 2 h posttreatment, the increase in binding is highly significant compared with controls; *P = 0.005.

### Fig. 8. Composite graphs showing changes in nuclear GEF and MEF2 (A) and changes in DNA binding at the GEF and MEF2 binding sites (B), respectively.
As suggested, GEF and MEF2 may be activated in the cytosol and translocate to the nucleus, or AMPK, after activation, may translocate to the nucleus where it then activates GEF through phosphorylation. ACC is a cytosolic protein and a known target of AMPK. Phospho-ACC protein levels increased twofold at 1 h post-AICAR injection, indicating that AMPK is active in the cytosol within 1 h post-AICAR treatment. The data showing an increase in phospho-AMPK at 5 h post-AICAR treatment, combined with the GEF phosphorylation, translocation, and binding within 1 h, suggest that activation of GEF is in the cytosol. Further support comes from previous findings from our laboratory showing an increase in GLUT4 gene transcription 3 h after an acute exercise bout (14) in rats. The increase in nuclear phospho-AMPK at 5 h may be a secondary response to AICAR treatment. On the basis of the time course of activation of AMPK in the cytosol (Fig. 3) at 1 h and the delayed increase of AMPK in the nucleus at 5 h (Fig. 4), we propose that AMPK phosphorylates GEF in the cytosol. GEF is then translocated to the nucleus with a maximum at 1 h.

When we consider the graphs in Fig. 8, A and B, there is a striking pattern. The increase in MEF2 translocation and binding to the GLUT4 promoter is preceded by the GEF response to AICAR treatment. This suggests that GEF, after phosphorylation and activation, may be recruiting MEF2 to the nucleus. This conclusion is supported by recent findings from the laboratory of Knight et al. (7) that MEF2A and GEF can associate and together regulate GLUT4 transcription. GEF and MEF2 coimmunoprecipitate in vitro and in vivo, and both must bind to their binding sites on the GLUT4 promoter region for activation of GLUT4 mRNA expression. Binding of only GEF or MEF2A alone does not significantly increase activity, whereas binding of both increases promoter activity over fourfold (7). Here, we report that AMPK can directly phosphorylate GEF, which in turn may lead to translocation to the nucleus and increased coactivation of MEF2 in DNA binding at the GLUT4 promoter region.

In summary, we have demonstrated that GEF is a substrate for AMPK. In addition, GEF and MEF2 protein increased in the nucleus at 2 h after AICAR treatment, suggesting an increase in translocation to the nucleus after AMPK activation. Both GEF and MEF2 have twofold increases in DNA binding by 2 h posttreatment. Together with previously established results on GLUT4 mRNA regulation by GEF and MEF2, this work further elucidates the pathway regulating GLUT4 mRNA expression and protein levels in skeletal muscle with AMPK activation.

REFERENCES