Regulation of GLUT4 expression in denervated skeletal muscle

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The total amount of GLUT4 protein in a cell is important for maximal glucose transport rates. Whole body glucose disposal and GLUT4 protein levels are correlated in humans (5, 20), with exercise training (10–12) and aging (13). Studies in transgenic mice show that the correlation between GLUT4 and glucose homeostasis is one of cause and effect (19). Mice overexpressing GLUT4 specifically in skeletal muscle have improved whole body insulin action, more rapid glucose clear-

ance from the blood, a higher rate of muscle glucose transport, and improved glucose homeostasis (15, 21, 35, 42, 43). Overexpression of GLUT4 in genetically diabetic ob/ob mice improved glycemic control and ameliorated diabetes (3, 7, 16). While decreased GLUT4 content is not responsible for insulin resistance, increased GLUT4 is capable of rescuing or preventing insulin resistance, and ways to increase GLUT4 content could be used to treat type II diabetes (49). For this reason, it is very important that we learn how GLUT4 expression is regulated, so we can develop therapies for treating diabetes.

Studies of how active muscle states, such as exercise, increase GLUT4 content have produced valuable information on how GLUT4 expression is regulated. Another way to gain information about GLUT4 gene expression is by studying the negative regulation of GLUT4 expression in inactive muscle states such as denervation.

Denervation by sciatic nerve resection decreases muscle GLUT4 mRNA and protein 50% 3 to 7 days after denervation (2, 4). Denervation of the rat hindlimb muscles leads to a reduction in both GLUT4 content and uptake of the nonmetabolizable glucose analog, 3-OMG (29), and loss of GLUT4 content is highly correlated with a reduction in insulin-stimulated glucose uptake (4). GLUT4 protein expression in denervated tissue appears to be dependent on changes in mRNA, which are regulated by decreased transcription rates of the GLUT4 gene (18).

Expression of GLUT4 is highly dependent on the transcription factors GLUT4 enhancer factor (GEF) and myocyte enhancer factor 2 (MEF2). GEF is the binding protein for the GLUT4 promoter element, Domain I. Domain I is the region between −742 bp and −712 bp upstream of the GLUT4 transcription initiation site (33). The MEF2-binding site is located between −473 bp and −464 bp of the GLUT4 gene promoter (41). Domain I and the MEF2-binding site cooperate to support the pattern of expression that endogenous GLUT4 follows (33). Regulated and tissue-specific expression of the GLUT4 gene occurs only in tissues in which sufficient GEF and MEF2 binding activity coincide. In differentiated muscle, MEF2 is required for GLUT4 mRNA expression (38, 41), and in mice expressing a reporter gene controlled by the GLUT4 gene promoter, mutation of the MEF2 binding domain was shown to result in a complete lack of reporter gene expression (6, 41). Confirming the imperative nature of MEF2 as a regulator of GLUT4 transcription, mutation of the MEF2 binding site in C2C12 cells also resulted in loss of function of the GLUT4 gene and decreased GLUT4 mRNA (22).

CaMK has been shown to be capable of activating MEF2. MEF2 proteins function in skeletal muscle as downstream
effects of calcium-regulated signaling pathways stimulated by motor nerve activity and its subsequent muscle contraction. In this way, MEF2 contributes to fiber-type-specific gene regulation (47, 48). CaMK allows MEF2 to become activated because CaMK phosphorylates inhibitory histone deacetylase (HDAC) proteins resulting in their dissociation from MEF2 and their export from the nucleus (8, 27, 44). Little is known about GEF regulation and whether GEF responds to calcium-signaling pathways.

Experiments were designed to test the hypothesis that decreased GLUT4 expression in denervated muscle occurs because of decreased CaMK activity in the noncontracting muscle. Decreased CaMK activity would then lead to depressed transcriptional activation by MEF2 and GEF. To test the role of calcium signaling in regulation of GLUT4 expression in denervated muscle, GLUT4 mRNA was measured in muscle of wild-type mice and mice expressing constitutively active CaMKIV.

To determine how GEF and MEF2 DNA binding activity change during denervation, we performed electrophoretic mobility shift assays using oligonucleotides corresponding to their cognate binding sites. Mice that express a lacZ reporter gene, expression of which is dependent on a gene promoter rich in MEF2 sequences, were also denervated to determine the ability of MEF2 to stimulate or repress gene expression in denervated muscle. To discover the responsive elements in the GLUT4 promoter, we denervated transgenic mice expressing the chloramphenicol acetyl transferase (CAT) reporter gene driven by different regions of the human GLUT4 promoter. We hypothesized that there would be no change in CAT expression after denervation in a transgenic mouse that did not have sufficient portion of the GLUT4 promoter to respond to denervation. In this study, new transgenic lines with promoter regions consisting of 895 bp of GLUT4 promoter minus deleted regions between −712 to −526 bp or −440 to −220 bp were denervated along with a line with 730 bp of promoter driving the expression of CAT. Each transgenic mouse line tested has different deleted portions of the GLUT4 promoter, but collectively, the mice have nearly the entire GLUT4 promoter deleted, or rendered ineffective, except for the MEF2 binding domain and the basal promoter (about 200 bp upstream of the initiation site).

MATERIALS AND METHODS

Mice. All procedures were approved by the Institutional Animal Care and Use Committee of East Carolina University. Mice were housed with room temperature and lighting controlled (20–22°C: 12:12-h light-dark cycle) and were given food and water ad libitum. Transgenic mice were identified by PCR using primers specific for their respective transgenes. The mice expressing constitutively active CaMKIV (CaMKIV-TG) (34) have been described previously. The MEF2/reporter mice were previously studied (31) and were graciously provided by Dr. Eric Olson (University of Texas Southwestern Medical Center). Mice expressing the CAT reporter gene controlled by different regions of the hGLUT4 gene promoter (hGLUT4-CAT) were produced at the Oklahoma Health Sciences Center and have been previously described (23, 32, 41). At the specified times after denervation, the mice were killed by CO2 asphyxiation, and the harvested muscles were weighed and stored at −80°C until use. Muscle phosphocreatine (PCr) was measured using a phosphorus magnetic resonance spectrometer. All procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina School of Medicine. The size of the protected fragments was determined by phosphorimager and quantitated with Imagequant software (Molecular Dynamics, Sunnyvale, CA). The size of the protected fragments was estimated by RNA transcripts labeled with [α-32P]UTP from Century Marker Templates (Ambion) by using T7 RNA polymerase. Endogenous GLUT4 and CAT measurements were normalized to GAPDH measurements. GAPDH was chosen as an appropriate normalizer for the data because of its lack of responsiveness to denervation.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays were done as described previously (33). Oligonucleotides were end-labeled with T4 polynucleotide kinase. Labeled probes (0.5 ng) were incubated with 10 mg of total protein isolated from muscle in a 10-ml reaction containing 2 mg of poly(dI-dC), 40 mM KCl, 5 mM MgCl2, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol for 20 min at room temperature. For competition studies, extracts were preincubated with various concentrations of unlabeled oligonucleotide, as indicated for 5 min before the addition of the radiolabeled probe. For competition studies using antibodies, extracts were preincubated with 2.5 mg of preimmune IgG, anti-GST-MEF2 IgG, or IgG from an irrelevant antiserum raised against a glutathione S-transferase (GST) fusion protein. Preincubation was carried out overnight in 1 ml of lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 5% glycerol, and 0.5% Nonidet P-40.
Quantitative RT-PCR. Quantitative RT-PCR (QRT-PCR) was used to analyze GLUT4 mRNA content in whole muscle samples from CaMKIV-transgenic mice, MEF2/reporter mice, and wild-type controls, according to the protocol described previously (30). After quantification of RNA, cDNA was synthesized with the reverse transcriptase reaction, using the iScript RT kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed using an ABI Prism 7300 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA). Prevalidated GLUT4 and lacZ primer/probe sets were purchased from PE Applied Biosystems. GLUT4 and lacZ mRNA values were normalized to 18s rRNA to correct for slight differences in sample substrate.

Nuclear isolation. Nuclear protein was isolated from gastrocnemius muscle using the NE-PER nuclear extraction kit (NE-PER; Pierce, Rockford, IL), as previously explained (25). Briefly, muscle samples were homogenized with 15 passes of a glass-on-glass tissue homogenizer in 10 volumes of cytoplasmic extraction reagent I. Cytoplasmic extraction reagent II was added at a final concentration of 5%, and the homogenate was spun in a centrifuge at 16,000 g for 5 min. The supernatant was extracted, and the pellet was resuspended in 100 μl of nuclear extraction reagent and sat on ice for 40 min with occasional mixing. The resuspended pellet was again spun in a centrifuge at 16,000 g for 10 min, and the supernatant, representing nuclear protein, was extracted and stored.

Protein sample preparation. Powdered muscle was homogenized (2 x 20 s) on ice in homogenization buffer containing 50 mM N-2-hydroxyethylpiperazine-N’-ethane (HEPES) (pH 7.4), 10 mM EDTA, 100 mM NaF, 50 mM Na pyrophosphate, 10 mM Na orthovanadate, 0.1 mg/ml aprotinin, 1% Triton, 5 μl/ml protease inhibitor cocktail, and 10 μl/ml phosphatase inhibitor cocktails I and II (Pierce) with an Ultra-Turrax homogenizer (IKA, Wilmington, NC). For measurement of total protein, samples were centrifuged, after homogenization, at 15,000 g for 10 min at 4°C. The supernatant was removed and stored on ice for protein quantitation.

For isolation of membrane-associated proteins, samples were centrifuged at 150,000 g for 1 h at 4°C. The pellet was resuspended by homogenization (2 x 10 s) on ice in homogenization buffer with an Ultra-Turrax homogenizer (IKA, Wilmington, NC). 1% IGEPAL was added, and samples were mixed by shaking at 4°C for 1 h. After a second centrifugation, the supernatant (particulate fraction) was collected and stored on ice for measurement of protein content followed by Western blot analysis. All samples were measured for protein content in triplicate by bicinchoninic acid procedure.

Western blot analyses. Proteins were resolved by SDS-PAGE on 7.5% or 10% Criterion resolving gels (Bio-Rad) at 200 V for 1 h in running buffer containing 25 mM Tris, 19.2 mM glycine and 0.1% SDS. Samples were then transferred at 100 V for 2 h to Immobilon-P polyvinylidene difluoride membrane in transfer buffer containing 25 mM Tris, 19.2 mM glycine. Membranes were then blocked with 5% nonfat milk with 1% BSA in 10 mM Tris-buffered saline (TBS) solution overnight at 4°C. The membranes were incubated for 1 h with primary antibody at room temperature in 5% nonfat milk/TBS or 5% BSA/TBS, according to antibody manufacturer protocol. Membranes were washed (4 x 5 min in TBST) and incubated with anti-rabbit-HRP or anti-goat-HRP conjugated antibodies in 5% milk/TBST at room temperature for 1 h. Membranes were incubated in Super Signal West Pico luminescence solution (Pierce), according to manufacturer protocol and exposed to film. Films were scanned using Epson Perfection 3200 negative flatbed scanner (Epson America, Long Beach, CA), and band density was quantified using GelPro Analyzer Software (Media Cybernetics, Silver Spring, MD).

Statistical analysis. For comparison of mRNA and protein content in control and denervated wild-type and transgenic mice, two-way ANOVA was used to identify statistical differences. An independent samples t-test was used for post hoc comparison of groups only after main effects were shown by two-way ANOVA. Results are reported as means ± SE. Comparison of control and denervated groups were performed by paired t-tests. All statistical analyses were performed with Statistical Package for the Social Sciences (SPSS; v. 11.0, Chicago, IL).

RESULTS

All mice included in this study were hindlimb denervated by sciatic nerve resection, which leaves the lower part of the hindlimb unable to contract. The contralateral sham-operated muscle served as the control. There was a main effect of denervation and expression of CaMKIV. Denervation in wild-type control mice resulted in a 68% decrease in GLUT4 mRNA in the plantaris muscle, and CaMKIV expression increased the amount of GLUT4 mRNA by 30% in the control muscle of transgenic mice (CaMK IV-TG Cont) compared with control muscle of wild-type mice (WT Cont) (Fig. 1). While expression of CaMK IV did not protect against the typical denervation response, as GLUT4 mRNA decreased by 54% (P = 5 x 10⁻⁵) in denervated muscle, expression of CaMK IV in denervated muscle was sufficient to cause GLUT4 mRNA to be 87% higher in transgenic vs. wild-type mice (P = 0.02).

The mechanism responsible for depressed GLUT4 expression is decreased gene transcription. Because binding of GEF and MEF2 to their respective binding domains on the GLUT4 promoter is necessary for normal GLUT4 transcriptional regulation (33), we tested the activity of GEF and MEF2 in denervated skeletal muscle by performing EMSAs to determine binding of GEF and MEF2 to their respective binding domains in the GLUT4 promoter region in gastrocnemius muscle. EMSAs for MEF2 and GEF were followed up by measuring nuclear and whole tissue (total) GEF and MEF2 protein in gastrocnemius muscle by Western blot analysis.

EMSA were performed to determine whether GEF binding changed after denervation. GEF binding decreased after denervation by 50% (P < 0.05) compared with GEF binding to Domain 1 in control muscles (Fig. 2). The nuclear and whole...
cell content levels of GEF after denervation in wild-type mice were then determined. Surprisingly, we found that denervation had no effect on nuclear GEF content (Fig. 3A), but GEF content in the whole tissue lysates (Fig. 3B) showed a strong trend for a 42% decrease (P < 0.077).

To investigate the role of MEF2 in the regulation of GLUT4 in response to denervation, MEF2A/D levels were measured in the nuclear and whole tissue protein extracts in the gastrocnemius after denervation. Nuclear content of MEF2A/D showed a strong trend toward being higher (129% increase; P = 0.079) in denervated muscle compared with control muscle (Fig. 4A) and total MEF2A/D protein was 125% more abundant (P = 0.03) in denervated muscle compared with control (Fig. 4B).

To determine the ability of MEF2 to stimulate transcription, transgenic mice developed to demonstrate activation of MEF2 activity were also denervated. These mice express a lacZ reporter gene controlled by a synthetic promoter containing three repeats of the MEF2 binding site (Fig. 5A). Measurement of β-galactosidase mRNA, the lacZ gene product, in gastrocnemius muscle by QRT-PCR after 72 h of denervation showed that there was no change in expression of the reporter gene after denervation (Fig. 5B). EMSA analysis was performed to test for MEF2 binding to the GLUT4 promoter in denervated gastrocnemius. EMSA analysis showed no change in MEF2 binding to the GLUT4 promoter in denervated vs. nondenervated muscle (Fig. 5C). These data both suggest that the ability of MEF2 to stimulate transcription is not impaired by denervation.

Another approach to investigate transcriptional regulation is to determine the DNA sequences of the promoter that are required for the denervation response. Using mice with reporter gene constructs expressing the CAT reporter gene con-
trolled by segments of the hGLUT4 promoter of different lengths, our laboratory has shown that there are no denervation-responsive elements above -895 bp from the transcription initiation site. To further understand what elements may be responsible for the large decrease in GLUT4 expression after denervation, hGLUT4-CAT reporter mice were used again to identify areas of the promoter that negatively influence GLUT4 in response to denervation. Mice were denervated and, after 72 h, gastrocnemius muscles were removed, and mRNA was isolated and prepared for measurement of CAT mRNA by QRT-PCR or RPA.

Mice with 895 bp of the hGLUT4 promoter controlling expression of the CAT reporter gene were denervated and mRNA was isolated from the gastrocnemius. CAT mRNA was measured by RPA and was found to be decreased by 57% ($P = 0.03$) in denervated muscle compared with controls (Fig. 6A).
When mice with 730 bp of hGLUT4 promoter were denervated and mRNA from the plantaris was measured by QRT-PCR, CAT reporter expression was found to be decreased by 93% \((P = 0.01)\) (Fig. 6B). This result demonstrates that the denervation responsive element is within the first 730 base pairs of the GLUT4 promoter. Because the GEF binding domain, Domain I, is disrupted in the 730 bp mice, the normal denervation response in muscle with this reporter construct indicates that the decrease in GLUT4 expression does not require that Domain I be intact.

Because deletion of the MEF2 or the GEF site results in loss of GLUT4 expression, we turned to two lines of mice that had 895 bp of the hGLUT4 promoter but with large deleted sections between known vital elements. The first of these new GLUT4-reporter mice has a deletion between bases \(-711\) and \(-526\) (Fig. 6C). These mice were denervated, and CAT reporter mRNA from the gastrocnemius was measured by RPA (Fig. 6D). Denervation decreased the amount of CAT mRNA in the gastrocnemius by 85% \((P = 4 \times 10^{-5})\). This demonstrates that the denervation responsive element does not likely exist between the MEF2 and GEF binding domains.

Another similar group of mice with the typical 895 bp GLUT4 promoter but with a deleted region between bases \(-220\) and \(-440\) was denervated (Fig. 6E). CAT reporter mRNA was measured from the gastrocnemius muscle of these mice by QRT-PCR, and they were found to have an 80% decrease in CAT mRNA \((P = 0.0001)\) (Fig. 6F). This result shows that the denervation response element is not likely within the promoter region between the MEF2 binding domain and the basal promoter.

**DISCUSSION**

The experiments of this study were designed to test the hypothesis that decreased GLUT4 expression in denervated skeletal muscle is due to depressed activity of CaMK, which then leads to decreased activation of the transcription factors MEF2 and GEF. To test the role of CaMK signaling in regulation of GLUT4 expression in denervated muscle, GLUT4 mRNA was measured in wild-type mice and mice expressing constitutively active CaMKIV. Normal CaMK signaling may not be accurately restored by expressing a constitutively active CaMK to make up for the loss of signaling through the diminished sporadically activated CaMK. However, we felt a loss of GLUT4 expression in denervated mice, with presumptively lower CaMK signaling, and an increase in GLUT4 in a mouse with constitutively active CaMK still made investigating the expression of GLUT4 in a denervated CaMK-transgenic mouse interesting for the purpose of determining whether increased CaMK signaling could attenuate the loss of GLUT4 expression in denervated muscle.

CaMKIV is not expressed in skeletal muscle and the major CaMK isoform in human and rodent muscle is CaMKII (1, 36, 37). CaMKII becomes active when bound by calcium/calmodulin and maintains its activity through autophosphorylation. Phosphorylation of CaMKII causes it to move to the nucleus. CaMKIV is typically located in the nucleus and becomes activated by calcium/calmodulin and by phosphorylation by CaMKK (39). We believe that CaMKIV may be a suitable surrogate for CaMKII because 1) CaMKII and CaMKIV share many common substrates, such as MEF2A and class II HDAC proteins, which seem likely to regulate MEF2 action, and thus GLUT4 expression (24, 27), 2) CaMKIV has been shown to be functional in skeletal muscle after it is expressed (46), 3) CaMKII is a large heteromeric protein, which because of its complexity, is much more difficult to study than the monomeric CaMKIV overexpression models (14). For this reason, we feel that mice that express constitutively active CaMKIV are good candidates for study of CaMK signaling in muscle (14), particularly in the study of GLUT4.

We expect that, had we been able to study mice with activated CaMKII, the results would have been similar to those observed in the CaMKIV model. CaMKII and CaMKIV share many common substrates, such as MEF2A and class II HDAC proteins, which seem likely to regulate MEF2 action, and thus GLUT4 expression (24, 27). In humans, one acute bout of exercise increased promoter binding of MEF2. MEF2A increased in the nucleus, but MEF2D did not (25). After one bout of acute swimming in rats, MEF2A binding to the GLUT4 promoter was increased. In CaMKIV-transgenic mice, ChIP assays were done to show that MEF2A binding to the GLUT4 promoter was increased. This study showed also that CaMKIV and CaMKII have MEF2A as a common substrate (40).

In other experiments, when we exercise trained these mice on a running wheel for 2 wk, there was a significant increase in GLUT4 in wild-type mice. CaMK-transgenic mice, however, had no increase in the amount of GLUT4 mRNA or protein in response to exercise. This suggests that CaMK stimulation in trained muscle plays a prominent role, since regulation by a pathway different than CaMK would be expected to generate an additive increase in GLUT4 content in exercised CaMK-transgenic mice. The results of this study provide evidence that CaMKIV activates the same signals as CaMKII and that these CaMK-dependent signals are of major importance in regulating GLUT4 expression. If CaMKIV and CaMKII had separate signaling pathways, then transgenic expression of CaMKIV and CaMK exercise would have had additive effects.

Control muscle of CaMK-transgenic mice had significantly more GLUT4 mRNA than control muscle of wild-type mice, but CaMKIV expression was not able to maintain GLUT4 expression levels in denervated muscle. Although there was a significant decline in GLUT4 mRNA content after denervation, there was significantly more GLUT4 in muscle of denervated CaMK-transgenic mice compared with wild-type mice. This is similar to previous work in our lab with AMPK (9). AMPK activation by AICAR administration was sufficient to increase the expression of GLUT4 in muscle, but AMPK was not able to maintain GLUT4 content when challenged with denervation. The increase in muscle GLUT4 content in exercised muscle is thought to result from increased activity of CaMK (unpublished data) and AMPK (reviewed in Ref. 45), although AMPK has been shown not to be required for the GLUT4 exercise response to occur (9). Supporting our hypothesis that CaMK causes increased production of GLUT4 after exercise is the finding that HDAC5, a CAMK-responsive transcriptional corepressor, inhibits GLUT4 expression (26), and the finding that, similar to exercise, CaMK increases the binding of MEF2A to the GLUT4 promoter. Although CaMK may mediate the exercise response of GLUT4, this study, in which mice expressing constitutively active CaMKIV had typical decreases in GLUT4 after denervation, has proven that the main cause for decreased GLUT4 content in denervated muscle is not de-
creased activity of CaMK. Megeney et al. (28) compared the difference in GLUT4 response to denervation and muscle inactivity. Glucose transport was decreased to the same extent in denervated muscles and muscles rendered noncontractile, but with the motor nerve still intact, by TTX superfusion of the sciatic nerve. However, GLUT4 content was decreased to a much greater extent in denervated muscles compared with TTX-treated muscles (28). These data indicated that denervation is a mixed model of muscle inactivity and elimination of neural signaling and delivery of neural trophic factors to the nerve, with the elimination of neural effects having a more profound role on GLUT4 content than muscle inactivity.

Having found that loss of CaMK signaling does not mediate the decrease in GLUT4 in denervated muscles, we investigated the content and activity of the putative GLUT4 transcription factors GEF and MEF2 to determine whether signaling regulating GLUT4 was mediated by GEF and MEF2. Binding of both GEF and MEF2 to their respective binding domains on the GLUT4 promoter is necessary for normal GLUT4 transcriptional regulation. EMSAs were performed to determine binding of GEF and MEF2 to their respective binding domains in the GLUT4 gene promoter. In addition, nuclear and whole tissue (total) GEF and MEF2 protein were determined by Western blot analysis. EMSAs suggested there was no change in MEF2 binding to the GLUT4 promoter. MEF2A/D protein tended to increase with denervation as denervated muscle had significantly higher total MEF2A/D and a trend (P = 0.08) for increased nuclear MEF2A/D protein. The lack of change in MEF2 binding and no change in MEF2 protein in the nucleus are consistent with a lack of change in nuclear HDAC5 (unpublished data) content in nuclei from denervated gastrocnemius.

The lack of regulation of MEF2 in denervation is also demonstrated by the results of an experiment with mice expressing an MEF2-dependent lacZ reporter gene. Reporter gene expression did not decrease after denervation, as it would have if MEF2 had taken on an inhibitory role in the denervated state. These MEF2 reporter mice have been used in the past to show that MEF2 transcription-enhancing activity increased in contracting muscle (48). The fact that these mice have been used to successfully show that MEF2 activity increases with muscle contraction seems to support our conclusion that the decrease in GLUT4 expression in denervated muscle is not due to decreased MEF2 activity, as indicated by the lack of decreased reporter gene expression in denervated muscle.

GEF binding to Domain I was measured and found to be decreased by 50% after denervation. Further evidence for decreased GEF activity was the finding that total GEF protein showed a similar trend (P = 0.07) with average total GEF 42% lower in denervated than in control tissue. However, the hypothesis that decreased GEF activation causes GLUT4 expression to be depressed seems unlikely given that hGLUT4/CAT reporter mice with 730 bp of GLUT4 promoter responded normally to denervation. With the position of Domain I being from −742 bp to −712 bp in the GLUT4 promoter, it is truncated in the mice with 730 bp of promoter, and this disrupts GEF binding. Intact Domain I is required for hormonal regulation, while complete deletion of Domain I abrogates GLUT4 expression. The truncated Domain I contained in the mice with 730 bp of promoter allows for basal GLUT4 regulation, but if signaling through GEF were responsible for decreased GLUT4 expression, then we would have expected that an intact Domain I would have to be present for proper repression of CAT reporter expression in response to denervation. Therefore, decreased GEF stimulation through the truncated Domain I may have played a role in the decline in expression of GLUT4.

To study GLUT4 regulation at the promoter, we focused on the regions that lie between Domain I and the MEF2 binding site and between the MEF2 binding site and the basal promoter. The combined effect of the battery of GLUT4/CAT reporter mice denervated was the deletion or mutation of nearly all of the GLUT4 promoter except for the MEF2 binding domain and the basal promoter. CAT reporter gene expression following denervation was significantly decreased in all transgenic lines. These data along with the fact that MEF2 was rejected as acting as an inhibitor to GLUT4 expression in denervated muscle due to the normal expression of the lacZ reporter gene in the MEF2-reporter mice led us to conclude that the denervation-induced decrease in GLUT4 gene expression is mediated through the truncated Domain I or the basal promoter region of the GLUT4 gene.

Previous data from our lab showed, similar to our findings with CaMK, that GLUT4 content does not decrease in denervated muscle due to a reduction in AMPK activity. However, mice with increased AMPK activity, due to AICAR treatment, had greater GLUT4 content after denervation than untreated controls. This finding is similar to our findings with CaMK in that mice expressing constitutively active CaMK IV had more GLUT4 after denervation than did denervated wild-type mice. Keeping in mind, as Megeney et al. (28) demonstrated, that denervation is a model of both muscle inactivity and abrogated neural signaling, we believe that these results demonstrate the ability of AMPK and CaMK to recover the loss of GLUT4 production due to muscle inactivity, but not the loss of GLUT4 due to decreased neural signaling and delivery of neural trophic factors. To elucidate this ability, future studies measuring GLUT4 in an innervated, but inactive, muscle expressing constitutively active CaMK or treated with AICAR should be performed.

In summary, the results of this study suggest that decreased CaMK activity does seem to be responsible for the portion of GLUT4 lost due to muscle inactivity, but more work will need to be done to determine the effectiveness of CaMK as a treatment for inactivity-induced reduction of GLUT4 content. Regulation by MEF2 does not seem to be involved in the denervation-induced loss of GLUT4 in muscle, while a role for GEF seems doubtful but cannot be ruled out yet. The effects, on GLUT4, of the decreased neural signaling are mainly mediated by factors other than CaMK, MEF2, and GEF. The decrease in GLUT4 in denervated muscle appears to be largely regulated by altered signaling at the basal promoter of the GLUT4 gene.

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