

Caffeine induces hyperacetylation of histones at the MEF2 site on the *Glut4* promoter and increases MEF2A binding to the site via a CaMK-dependent mechanism

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Mukwevho E, Kohn TA, Lang D, Nyatia E, Smith J, Ojuka EO. Caffeine induces hyperacetylation of histones at the MEF2 site on the *Glut4* promoter and increases MEF2A binding to the site via a CaMK-dependent mechanism. *Am J Physiol Endocrinol Metab* 294: E582–E588, 2008. First published January 15, 2008; doi:10.1152/ajpendo.00312.2007.—This study was conducted to explore the mechanism by which caffeine increases GLUT4 expression in C₂C₁₂ myotubes. Myoblasts were differentiated in DMEM containing 2% horse serum for 13 days and the resultant myotubes exposed to 10 mM caffeine in the presence or absence of 25 μ M KN93 or 10 mM dantrolene for 2 h. After the treatment, cells were kept in serum-free medium and harvested between 0 and 6 h later, depending on the assay. Chromatin immunoprecipitation (ChIP) assays revealed that caffeine treatment caused hyperacetylation of histone H3 at the myocyte enhancer factor 2 (MEF2) site on the *Glut4* promoter ($P < 0.05$) and increased the amount of MEF2A that was bound to this site ~ 2.2 -fold ($P < 0.05$) 4 h posttreatment compared with controls. These increases were accompanied by an ~ 1.8 -fold rise ($P < 0.05$ vs. control) in GLUT4 mRNA content at 6 h post-caffeine treatment. Both immunoblot and immunocytochemical analyses showed reduced nuclear content of histone deacetylase-5 in caffeine-treated myotubes compared with controls at 0–2 h posttreatment. Inclusion of 10 mM dantrolene in the medium to prevent the increase in cytosolic Ca²⁺, or 25 μ M KN93 to inhibit Ca²⁺/calmodulin-dependent protein kinase (CaMK II), attenuated all the above caffeine-induced changes. These data indicate that caffeine increases GLUT4 expression by acetylating the MEF2 site to increase MEF2A binding via a mechanism that involves CaMK II.

myocyte enhancer factor 2; glucose transporter 4; histone deacetylase; chromatin immunoprecipitation assay; Ca²⁺/calmodulin-dependent protein kinase II; histone H3

REGULAR EXERCISE INCREASES THE CONTENT of the GLUT4 glucose transporter that increases glucose disposal and protects against type II diabetes (8, 28). There is mounting evidence that the increase in cytosolic Ca²⁺, triggered by each wave of depolarization during muscle contraction, may be one of the signals that cause GLUT4 upregulation in response to exercise. Previous reports (25, 26) have shown that increasing cytosolic Ca²⁺ with the use of caffeine upregulated GLUT4 expression in L6 myotubes. The increase in GLUT4 content due to caffeine was abolished when Ca²⁺ release from the sarcoplasmic

reticulum was blocked by dantrolene or when Ca²⁺/calmodulin-dependent protein kinase (CaMK) II activity was inhibited by KN93. These results suggested that GLUT4 expression might be regulated by Ca²⁺ via CaMK signaling. This suggestion seemed reasonable in light of the numerous observations that GLUT4 is upregulated by exercise (19, 24, 27, 28), which also increases cytosolic Ca²⁺ levels and CaMK II activity (31, 33). Recently, using C₂C₁₂ myotubes that over-expressed constitutively active or dominant-negative CaMK IV, we observed that increased CaMK activity upregulated the binding of myocyte enhancer factor 2 (MEF2)A to the *Glut4* promoter (31). Results from these studies provide strong support for the hypothesis that the increase in skeletal muscle GLUT4 expression in response to caffeine is mediated via a CaMK-induced increase in the binding of MEF2A to the *Glut4* gene. However, the mechanism by which CaMK signals to increase MEF2A binding to the *Glut4* promoter remains to be elucidated.

Access of transcription factors to their binding domains on DNA is a well-regulated process requiring the action of histone-modifying enzymes. Core histone proteins, which control gene expression by modulating the structure of chromatin, may be acetylated at lysine residues by histone acetyl transferases (HATs) to relax chromatin or deacetylated by histone deacetylases (HDACs) to condense it (2). In general, genes are expressed when the chromatin surrounding them is hyperacetylated so that transcription factors (e.g., MEF2) have access to their binding domains on DNA. Conversely, genes are repressed when the chromatin is hypoacetylated (3, 32). HATs and HDACs also interact with sequence-specific DNA binding factors, such as transcription factors, to confer target gene specificity to their actions (2). In this regard, MEF2 proteins have been shown (10, 20) to associate directly with class II HDACs via an 18-amino acid motif to form a complex on regulatory elements of genes harboring MEF2 binding sites, resulting in repression of those genes. HATs, such as p300, have also been shown (29) to interact with the same MEF2 domain, but interaction of HDACs and HATs with MEF2 is mutually exclusive. External signals (e.g., stress or exercise) cause class II HDACs (e.g., HDAC5) to shuttle from the nucleus to the cytoplasm (5, 18). The nucleocytoplasmic shuttling of HDACs, which is dependent on phosphorylation of two

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serine-containing motifs on the deacetylases by CaMKs (7, 12, 16), relieves HDAC-mediated repression of MEF2-dependent genes by promoting the association of HATs with MEF2 (11, 34).

Because caffeine activates CaMK II by releasing Ca^{2+} from the sarcoplasmic reticulum (13), we hypothesized that the caffeine-induced increase in *Glut4* gene expression occurs via a CaMK-mediated hyperacetylation of the MEF2 site on the gene and increases MEF2A binding to the site. To test this hypothesis, we incubated C₂C₁₂ myotubes with caffeine in the presence or absence of dantrolene or KN93 and measured nuclear HDAC5 abundance, acetylation of histone H3 in the neighborhood of the MEF2 site on the *Glut4* promoter, and the extent to which MEF2A was bound to the site. We found that caffeine reduced the content of HDAC5 in the nucleus, induced hyperacetylation of histone H3 in the region surrounding the MEF2-binding site, and increased MEF2A binding to the *Glut4* gene. Dantrolene or KN93 attenuated these effects of caffeine.

EXPERIMENTAL PROCEDURES

Materials. C₂C₁₂ myocytes were kindly donated by Kathryn H. Myburgh (University of Stellenbosch). Cell culture plates and flasks were purchased from Greiner Bio-One (Frickenhhausen, Germany) and culture medium and reagents from Gibco. KN93 and dantrolene were obtained from Sigma-Aldrich (St. Louis, MO), protein assay kit from Bio-Rad (Hercules, CA), enhanced chemiluminescence reagents from Amersham Laboratories (Cape Town, South Africa), and MG132 from Calbiochem. Antibodies against α -tubulin and MEF2A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-HDAC5 from Cell Signaling Technology (Danvers, MA). Goat anti-mouse (Alexa) fluor 488 and donkey anti-rabbit cyanine-3 (Cy3) fluor 546 secondary antibodies and 4,6-diamidino-2-phenylindole (DAPI) nuclear stain were from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody and anti-mouse IgG were purchased from Cell Signaling Technology. The Zeiss AxioVert 200M microscope using Axiovision software was used for immunocytochemistry analysis. Density of signals in Western blots was determined using UVIssoft software.

Cell Culture. C₂C₁₂ myocytes were maintained at 37°C in 5% CO₂ on 100-mm collagen coated plastic petri dishes containing DMEM supplemented with 10 mM creatine, 100 $\mu\text{U}/\text{ml}$ streptomycin, 100 $\mu\text{U}/\text{ml}$ penicillin, 0.25 $\mu\text{g}/\text{ml}$ fungizone and heat-inactivated 10% fetal calf serum. Cells were maintained in continuous passage by trypsinization of subconfluent cultures with 0.25% trypsin EDTA. Myoblast differentiation was induced by introduction of medium containing 2% heat-inactivated horse serum when myocytes were ~80% confluent. Cells were kept in this medium for ~13 days until myotubes were well formed. The resultant myotubes were incubated in serum-free medium containing caffeine in the presence or absence of 25 μM KN93 or 10 mM dantrolene for 2 h and harvested 0–6 h later.

Immunocytochemical analysis of myotubes. Myotubes were kept in serum-free medium for ~12 h before treatments. The treatments involved incubation in medium containing 10 mM caffeine in the presence or absence of 10 mM dantrolene or 25 μM KN93 for 2 h. Immediately after treatments, myotubes were washed in ice-cold PBS and fixed with 3:1 methanol/glacial acetic acid at –20°C for 10 min. Thereafter, they were washed in PBS, blocked in 1% BSA for 30 min, and incubated overnight at 4°C with antibodies against HDAC5 and MEF2A. The following day, cells were washed and incubated with fluorochrome-conjugated anti-rabbit (Cy3) and goat anti-mouse (Alexa) secondary antibodies (diluted 1:1,000) for 2 h at room temperature. Nuclei were stained using a 1:50 dilution of 0.05 $\mu\text{g}/\text{ml}$ DAPI for 10 min at room temperature. After being washed, a coverslip was

mounted on the cells with a solution consisting of 40% glycerol, 20% Mowiol, 8% n-propyl gallate, and 0.2 M Tris buffer (pH 7.4). Subcellular location of HDAC5 and MEF2A was viewed using a Zeiss Axiovert 200M fluorescence microscope equipped with plan-neofluar optics set at 15 (Cy3), 10 (Alexa 488), and 1 (DAPI) as well as an AxioCam HR charge-coupled device camera. Adobe Photoshop 7 software was used for image processing.

Nuclear extracts. C₂C₁₂ myotubes were treated as above, being washed twice with ice-cold PBS, and 300 μl homogenization buffer (10 mM Tris, 1 mM EDTA, 5 mM MgCl₂, 1.4 M sucrose, 10 mM Na₄P₂O₄, 20 mM NaF, 0.15 μM okadaic acid, 4 mM Na₃VO₄, 1 \times complete protease inhibitor, 30 μM MG132, pH 7.4) was added. After being scraped loose from the plate, myotubes were homogenized with a Dounce homogenizer and the homogenate centrifuged at 600 g for 10 min at 4°C. The supernatant containing the crude cytosolic fraction was centrifuged for a second time at the same speed for 5 min to produce the final cytosolic extract. The pellet containing nuclei was washed in 300 μl homogenization buffer, centrifuged as above, and resuspended in 75 μl of nuclear extraction buffer (25% glycerol, 0.2 mM EDTA, 15 mM MgCl₂, 20 mM HEPES, 450 mM NaCl, 0.5% Triton X, 100 mM KCl, 25 μM MG132, 4 mM Na₃VO₄, 20 mM NaF, 1.5 μM okadaic acid, 10 mM Na₄P₂O₇, 1 \times complete protease inhibitors). The nuclear fraction was sonicated on ice for 5 s and centrifuged at 600 g for 5 min, and the supernatant (nuclear extract) was collected. Protein concentrations of the extracts were measured using the Bio-Rad protein assay. To determine whether the extraction protocol effectively separated cytosolic and nuclear proteins, the activity of lactate dehydrogenase (LDH), a cytosolic protein, and the contents of α -tubulin (cytosolic protein) and histone H3 (nuclear protein) were measured in both extracts. LDH activity was measured spectrophotometrically by assessing the rate at which NADH was oxidized to NAD⁺ in an LDH-catalyzed reaction that is coupled to the conversion of pyruvate to lactate. Fifteen micrograms of cytosolic or nuclear extracts was added to 1 ml of reaction buffer [50 mM Tris (pH 7.5), 4 mM EDTA, 2 mM pyruvate, and 0.14 mM NADH] and the absorbance measured at 340 nm for ~1 min. The contents of α -tubulin and histone H3 proteins were measured by Western blot, as described below.

Western blots. Proteins from nuclear and cytosolic extracts were solubilized in Laemmli sample buffer, separated on 7.5% gel by SDS-PAGE, and transferred to PVDF membrane as described earlier (26). Membranes were blocked and incubated with antibodies against MEF2A, HDAC5, histone H3, or α -tubulin as recommended by manufacturers. Membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, and proteins were detected using enhanced chemiluminescence. The HDAC5/MEF2A ratio in nuclear extracts was calculated and normalized to controls and graphed to show the effect of different treatments on their relative nuclear abundance.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (31). Briefly, 4 h after a single 2-h treatment (see above), cells were cross-linked with 1% formaldehyde and lysed on ice. Chromatin was sheared to fragments ~300–1,000 bp by sonication and centrifuged, and the supernatant (containing chromatin fragments) was precleared with salmon sperm DNA/protein A agarose to yield input sample. Chromatin from 250 μl of the input sample was immunoprecipitated by incubation with protein agarose A and antibodies directed against MEF2A, acetylated histone H3, or histone H3 or with immunoglobulin G (IgG). Precipitated complexes were reverse cross-linked in 0.5 M NaCl at 65°C for 6 h, and the coimmunoprecipitated DNA was purified by phenol-chloroform extraction and resuspended in dH₂O. A 270-bp fragment corresponding to nucleotides –336 to –604 of the mouse *Glut4* promoter containing the MEF2 binding site was amplified by PCR using the following primers (+MEF2 site primers): forward 5'-CAG GCA TGG TCT CCA CAT ACA C-3'; reverse 5'-GGT AAC TCC AGC AGG ATG ACA-3'. A 315-bp fragment

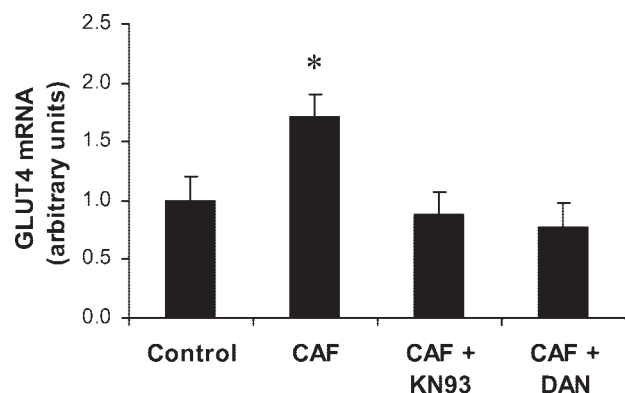


Fig. 1. Glucose transporter 4 (GLUT4) mRNA is increased by caffeine (CAF) in C_2C_{12} myotubes. Well-differentiated myotubes were incubated with 10 mM CAF in the presence or absence of 25 μ M KN93 or 10 mM dantrolene (DAN) in serum-free medium for 2 h every 12 h for 2 days. Myotubes were harvested 6 h after the last treatment. GLUT4 mRNA was measured by real-time PCR as described in EXPERIMENTAL PROCEDURES. * $P < 0.05$ vs. control.

corresponding to nucleotides +2,868 to +3,183 (relative to the start of transcription), which does not contain the MEF2 site, was used as negative control. This site was amplified using the following primers (–MEF2 site primers): forward 5'-CCA ACA GCT CTC AGG CAT

CAA-3'; reverse 5'-CCA TTC CAC AGG CAA GCA G-3'. DNA from 2.5 μ l of input sample that did not undergo ChIP but was reverse cross-linked and purified identically, as described above, was also PCR amplified using the same set of primers. PCR products from the immunoprecipitated chromatin and from corresponding inputs were separated by electrophoresis on 2% agarose gels. The gels were stained with ethidium bromide and the densities of the bands quantified.

Measurement of GLUT4 mRNA. Well-differentiated myotubes were serum starved overnight and incubated with 10 mM caffeine in the presence or absence of 25 μ M KN93 or 10 mM dantrolene. Myotubes were harvested 6 h later and RNA isolated using 1 ml of TRI Reagent according to the manufacturer's instructions (Ambion). RNA quantity was assayed by measuring the absorbance at 260 nm, and the integrity of the RNA was checked by running samples on a 1% formaldehyde agarose gel. Genomic DNA was eliminated from the samples by digestion with DNase 1 for 90 min at 37°C. DNase 1 was subsequently deactivated by incubation at 75°C for 5 min. cDNA was synthesized from 1 μ g of total RNA using 200 U of Moloney murine leukemia virus reverse transcriptase (Promega) for 1 h in a reaction mixture containing 2 mM dNTPs, 2.5 mM $MgCl_2$, 20 U RNasin, and 1 \times Promega RT buffer. Real-time PCR was performed in triplicate on the cDNA in a Light Cycler PCR machine (Roche) using SYBR Green PCR reagents from Roche and primers that amplify a 270-bp region in the mouse *Glut4* gene (forward 5'-

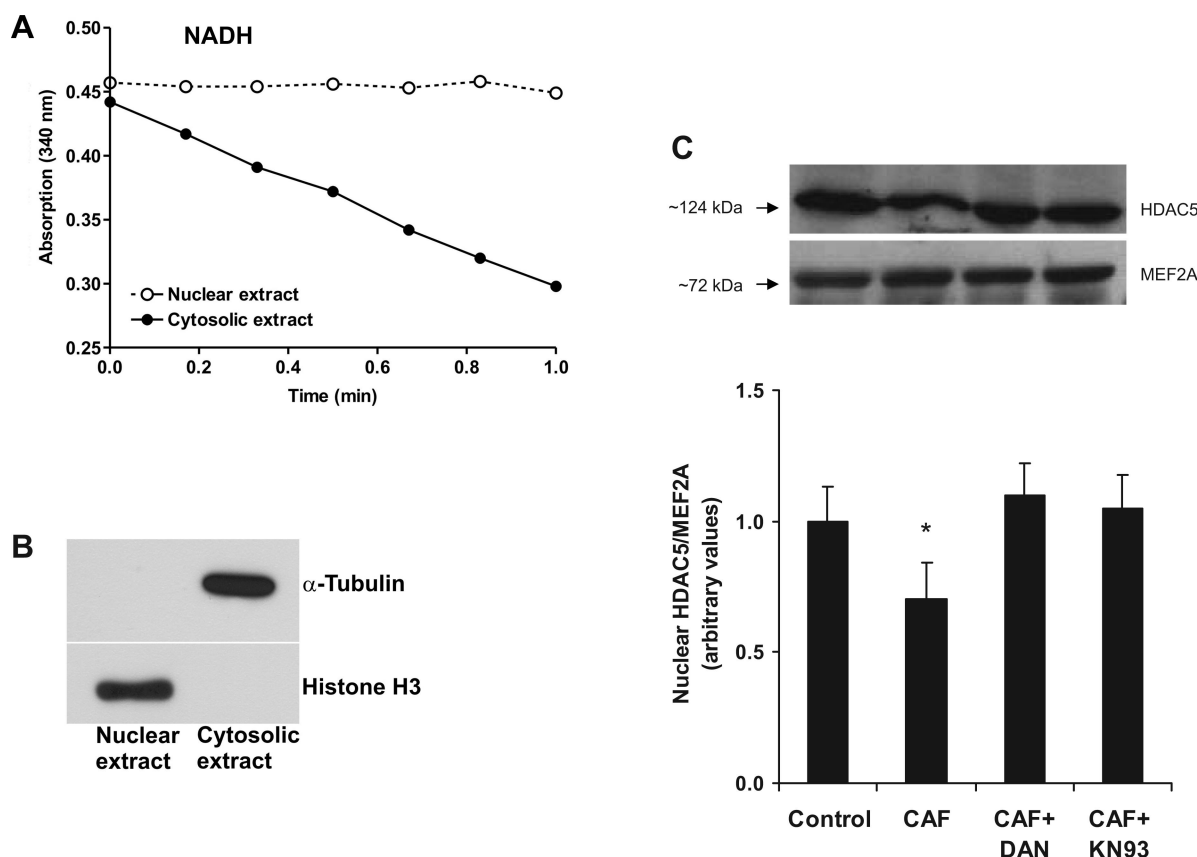


Fig. 2. Nuclear and cytosolic contents of myocyte enhancer factor 2A (MEF2A) and histone deacetylase-5 (HDAC5). Myotubes were treated with 10 mM CAF in the presence or absence of 10 mM DAN or 25 μ M KN93 for 2 h, incubated in serum-free medium for 2 h, and harvested. Cytosolic and nuclear proteins were separated as described in EXPERIMENTAL PROCEDURES. MEF2A and HDAC5 levels were measured by Western blot from the nuclear extract. A: lactate dehydrogenase activity is evident in the cytosolic fraction but absent in the nuclear fraction. B: α -tubulin (a cytosolic protein) is detectable in the cytosolic fraction but not in the nuclear fraction, whereas histone H3 (a nuclear protein) is enriched in the nuclear fraction but absent in the cytosolic fraction. Collectively, these control experiments show that the nuclear extraction protocol was effective. C: representative Western blots of MEF2A and HDAC5 from nuclear extracts and a histogram of the HDAC5/MEF2A ratio for the various treatments. The histogram reflects the abundance of HDAC5 relative to MEF2A in the nucleus. * $P < 0.05$ vs. control; $n = 5$ independent experiments.

GCAGCGAGTGACTGGAACA-3'; reverse 5'-CCAGCCACGTTG-CATTGTAG-3'). Relative mRNA concentrations were calculated using the PCR threshold cycle according to the method described by Livak and Schmittgen (15). Mean values were corrected to mouse GAPDH (forward 5'-GCCTGCTTACCACCTTC-3'; reverse 5'-GGCTCTCCAGAACATCATCC-3') and ribosomal S12 (forward 5'-GGAAGGCATAGCTGCTGGAGGTGT-3'; reverse 5'-CGATGACATCCTTGGCCTGAG-3') genes (internal controls) and expressed relative to a control in each experiment.

Statistics. Data are presented as means \pm SD. Statistical differences between treatments were determined using a one-way ANOVA or Student's *t*-test as appropriate. Significance was accepted at $P < 0.05$. When the ANOVA test showed a significant difference, post hoc analysis was performed using Fisher's least significant difference test. STATISTICA 7 software was used for these analyses.

RESULTS

GLUT4 mRNA is increased by caffeine. C₂C₁₂ myotubes were treated with 10 mM caffeine or vehicle for 2 h and

harvested 6 h later for GLUT4 mRNA measurement. Addition of 10 mM caffeine to the medium increased GLUT4 mRNA ~ 1.8 -fold ($P < 0.05$; Fig. 1). The caffeine-induced increase in GLUT4 mRNA was abolished by dantrolene or KN93. Similar results have been reported previously (26) regarding GLUT4 protein.

Caffeine decreases nuclear HDAC5 content. After C₂C₁₂ myotubes were treated with 10 mM caffeine in the presence or absence of 10 mM dantrolene or 25 μ M KN93 for 2 h, cytosolic and nuclear proteins were separated as described in EXPERIMENTAL PROCEDURES. LDH activity was detectable in the cytosolic fraction but not in the nuclear fraction as expected (Fig. 2A). Likewise, α -tubulin was seen in the cytosolic but not nuclear fraction. In contrast, histone H3 was predominantly found in the nuclear fraction (Fig. 2B). These observations indicate that our extraction protocol effectively separated cytosolic and nuclear proteins. In nuclear extracts, caffeine reduced HDAC5 ($P < 0.05$) but not MEF2A content compared

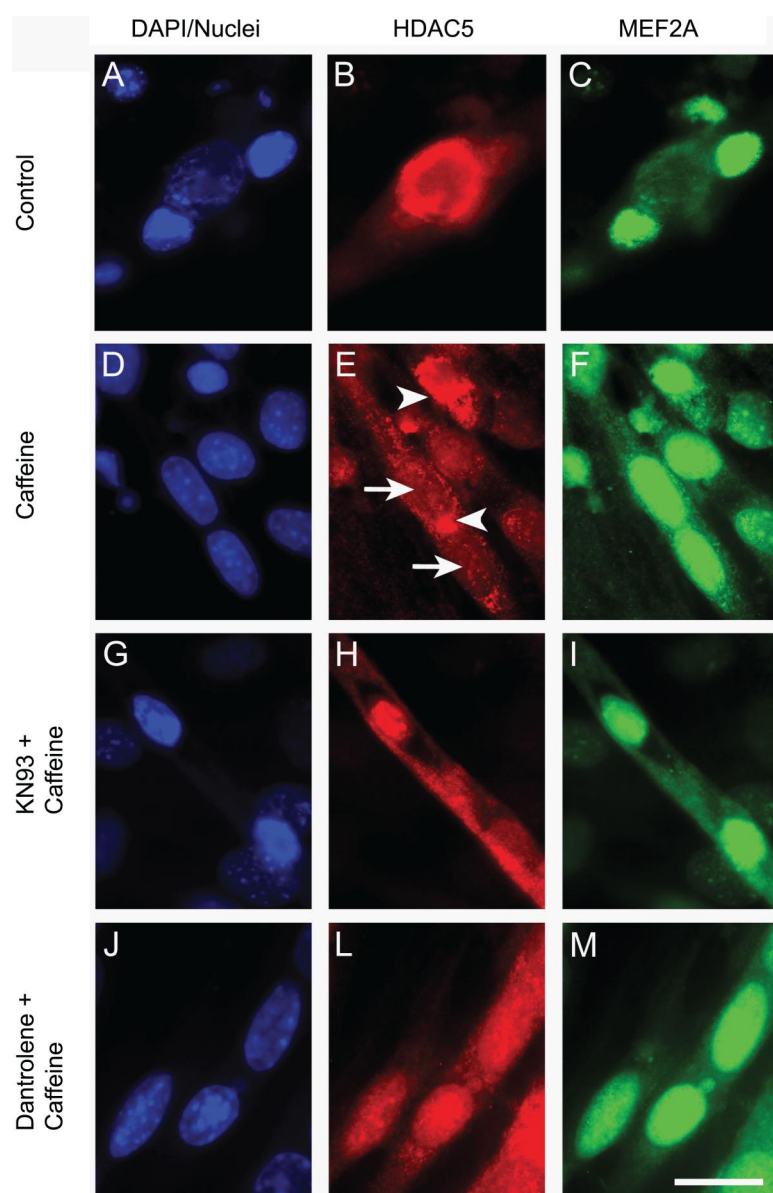


Fig. 3. Representative immunocytochemical images of C₂C₁₂ myotubes. After incubation in medium containing 10 mM CAF in the presence or absence of 10 mM DAN or 25 μ M KN93 for 2 h, subcellular localization of the nucleus, HDAC5, and MEF2A was determined using the immunocytochemical technique described in EXPERIMENTAL PROCEDURES. A–C: control untreated myotubes. D–F: myotubes incubated with 10 mM CAF. G–I: myotubes treated with CAF + 25 μ M KN93. J–M: myotubes incubated with CAF + 10 mM DAN. Blue, 4,6-diamidino-2-phenylindole (DAPI) nuclear stain; red, HDAC5; green, MEF2A. Arrows indicate decreased intranuclear HDAC5 and arrowheads increased perinuclear HDAC5 levels. Scale bar, 10 μ m.

with untreated controls (Fig. 2C). Consequently, HDAC5/MEF2A ratio was decreased by caffeine. Inclusion of dantrolene or KN93 together with caffeine attenuated the decreases in nuclear HDAC5 content and HDAC5/MEF2 ratio. These results indicate that caffeine causes HDAC5 export from the nucleus via a calcium-dependent CaMK II mechanism.

Immunocytochemical analysis confirms that HDAC5 nuclear export occurs via calcium and CaMK II-dependent mechanism. Immunocytochemistry with an antibody directed against MEF2A was performed and revealed that MEF2A was located predominantly in the nucleus in untreated cells or when treated with caffeine in the absence or presence of dantrolene or KN93 (Fig. 3). In contrast, HDAC5 was found in both the nucleus and the cytosol in untreated cells. Incubation with caffeine caused a decrease in intranuclear but an increase in perinuclear HDAC5 density (Fig. 3E). Preincubation with KN93 or dantrolene prior to caffeine administration blocked the effect of caffeine (Fig. 3, G–I and J–M). These representative images, which are consistent with the observation from Western blots, show that caffeine causes HDAC5 to traffic out of the nucleus in a calcium- and CaMK II-dependent manner.

Caffeine induced hyperacetylation of histone H3 at the MEF2 site on the *Glut4* promoter and increased the binding of MEF2A to the site. ChIP assays were performed to determine the extent of histone acetylation and MEF2A binding at the

MEF2 site on the *Glut4* promoter in C₂C₁₂ myotubes. Representative agarose gels showing PCR products from various treatment groups (control, caffeine, caffeine + KN93, caffeine + dantrolene, KN93 only, or dantrolene only) following immunoprecipitation with MEF2A, acetyl histone H3, or total histone H3 antibodies are shown in Fig. 4A. Signals from input sample, representing the total MEF2 site in the sample, and from immunoprecipitated samples, showing the fraction that is immunoprecipitated with the respective antibody, are shown adjacent to each other (for each treatment group) to facilitate comparison. The fraction, immunoprecipitated MEF2 site/total MEF2 site, which represents the proportion of bound MEF2A and the levels of acetylation of the MEF2 site under different treatments, is graphed in Fig. 4, C and D. Figure 4C shows that caffeine treatment increased the amount of MEF2A that was bound to its *cis* element, but the effect of caffeine was abolished when dantrolene or KN93 was in the medium. Likewise, the level of acetylation of histone H3 at the MEF2 site was increased by caffeine compared with controls, but the increase was abolished by KN93 or dantrolene.

Because the different treatments may remodel chromatin, it may be argued that the observed differences in signal intensities from the various treatments were due to inequalities in cross-linking efficiencies or antibody accessibility to histones. Therefore, we performed control experiments using an anti-

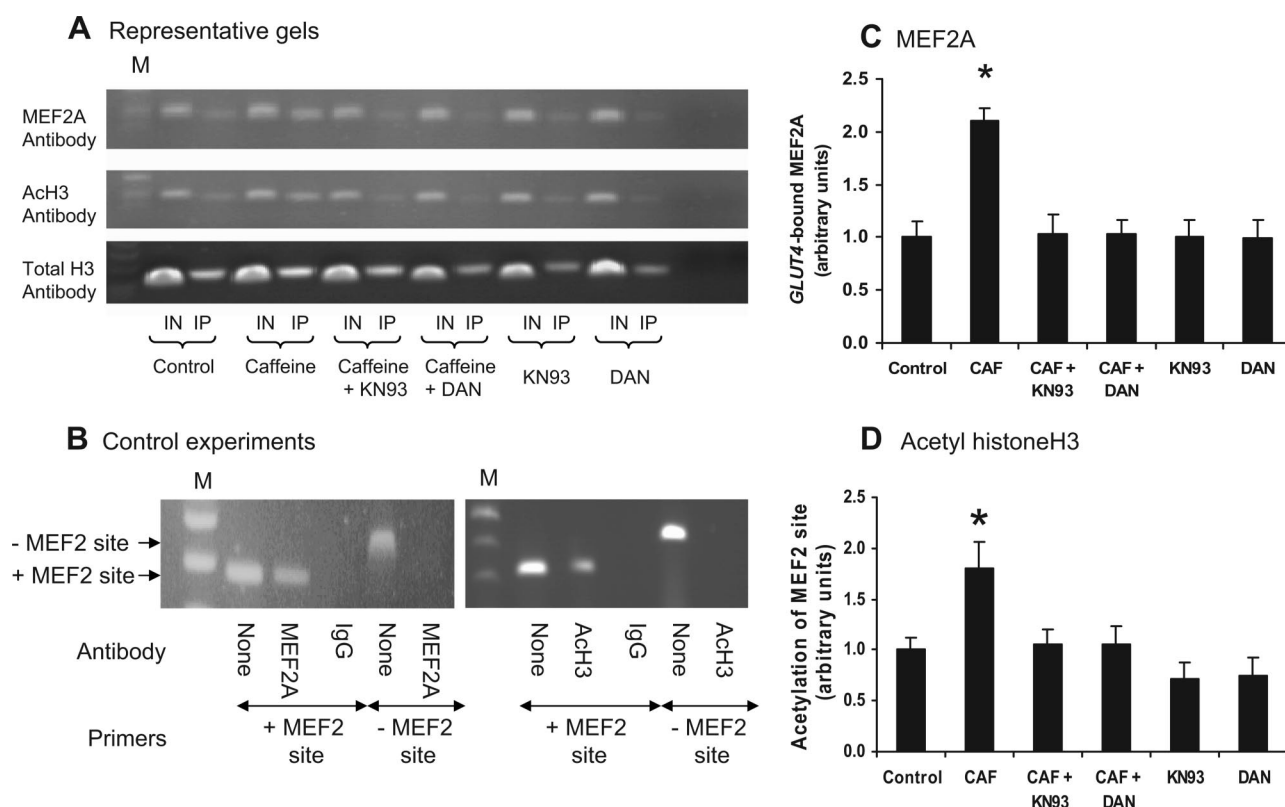


Fig. 4. CAF induced hyperacetylation of histone H3 at the MEF2 site on the *Glut4* promoter and increased the binding of MEF2A to the site. Myotubes were treated with 10 mM CAF in the presence and absence 10 mM DAN or 25 μ M KN93 for 2 h. The level of acetylation of histone H3 at the MEF2 binding site on the *Glut4* promoter and the extent of MEF2A binding to this site were determined by chromatin immunoprecipitation assay 4 h postincubation. A: a section of the *Glut4* promoter spanning the MEF2 binding site was PCR amplified from DNA that was coimmunoprecipitated (IP) with acetylated histone H3 (AcH3), MEF2A, or histone H3 antibody and from corresponding input (IN) samples. B: control experiments including primers that recognize a region of the *Glut4* gene that does not contain the MEF2 site (–MEF2 site primers) and using mouse immunoglobulin G (IgG), which does not bind MEF2A or histone H3 proteins. C: relative amounts of *Glut4* promoter-bound MEF2A calculated from signal densities from PCR reactions. The bars represent the IP/IN ratio normalized to control. D: relative amounts of acetylated histones at the MEF2 site on the *Glut4* gene. The bars represent the IP/IN ratio normalized to control. * $P < 0.05$ vs. control. M, molecular weight marker.

body against total histone H3. As can be seen from Fig. 4A (total H3), the signal intensity was similar in all treatments when this antibody was used, demonstrating that there were no differences in cross-linking efficiencies and/or antibody accessibility. Additional control experiments (Fig. 4B) using primers that recognize a region of the *Glut4* gene, which does not contain the MEF2 site (–MEF2 site primers), and using mouse IgG, which does not bind MEF2A or histone H3 proteins, were conducted to demonstrate that the ChIP assays 1) detected MEF2A that bound specifically to its site on the *Glut4* gene and 2) used antibodies (against MEF2A and acetyl histone H3) that interacted specifically with their respective antigens. Collectively, our results are consistent with the interpretation that caffeine increases the binding of MEF2A to the *Glut4* promoter, via increased acetylation of the MEF2 site, by a mechanism that involves calcium and CaMK.

DISCUSSION

It has been shown previously (26) that caffeine increased GLUT4 content in cultured L6 myotubes by a mechanism that depends on CaMK activity. The aim of the present study was to further explore the mechanism by which CaMK activation by caffeine upregulates GLUT4 expression. We report here that 10 mM caffeine increases the binding of MEF2A to its binding domain on the *Glut4* gene and upregulates GLUT4 expression in differentiated C₂C₁₂ myotubes. The increases in MEF2A binding to the *Glut4* gene and GLUT4 mRNA content were abolished when CaMK activity was inhibited by KN93, suggesting that caffeine acts at the transcriptional level via a mechanism that requires CaMK activity. Others (16, 20) have reported previously that CaMK phosphorylates class II HDACs and causes their nuclear efflux. It is conceivable that HDAC nuclear export, which also occurred in response to caffeine administration in the present study, would give rise to increased nuclear HAT activity and promote chromatin relaxation and increased accessibility of MEF2 factors to their binding domains on DNA. In support of this notion, we found increased acetylation of histone H3 in the neighborhood of the MEF2 site on the *Glut4* promoter after caffeine treatment compared with controls. The fact that these histones were not hyperacetylated in samples that had been treated with KN93 confirms that acetylation of histones at the MEF2 site on the *Glut4* promoter is regulated by CaMK. Of note is the observation (17, 30) that HATs such as p300 also interact directly with MEF2 factors to enhance binding activity. Ma et al. (17) recently demonstrated that MEF2C can be acetylated by p300 at multiple lysine residues and that acetylation increased MEF2C binding activity. The fact that acetylatable lysine residues on MEF2 factors are fully conserved in MEF2A, MEF2C, and MEF2D across several species suggests that MEF2 acetylation by p300 (or other HATs) at these sites is a general mechanism. Because the acetylatable lysines are not found on the NH₂-terminal DNA binding domain of MEF2, it has been suggested (4, 9) that the observed increase in binding activity due to acetylation occurs because of a conformational change in MEF2. These observations raise the possibility that the increase in MEF2A binding due to caffeine may also be due to MEF2A acetylation. Work is in progress in our laboratory to assess this hypothesis.

McKinsey et al. (20) reported that CaMK activation disrupts MEF2/HDAC complexes that are bound to specific genes and removes the repression conferred by the associated HDACs on the gene. Although it remains to be demonstrated that CaMK signaling reduces the content of MEF2A/HDAC5 on the *Glut4* gene, our observation that GLUT4 transcription increased with caffeine administration in a manner that was both CaMK dependent and negatively correlated with HDAC5 content supports the hypothesis that the *Glut4* gene might be regulated by the mechanism described by McKinsey et al. (20). Czuby et al. (6) recently provided data in support of the notion that GLUT4 expression is regulated by HDAC5. They showed that expression of a mutant form of HDAC5, which binds and represses MEF2 factors but cannot be exported from the nucleus, decreased GLUT4 expression in mouse cardiac muscle. Because caffeine was able to increase GLUT4 mRNA in the present study, it may be assumed that it activates both binding and transactivation domains of MEF2A. However, the mechanism by which caffeine increased MEF2A transactivation remains to be investigated. Because the MAP kinases p38 and ERK5 interact with and activate the transcriptional domain of MEF2 factors (21–23), the possibility exists that caffeine also activates these signaling molecules. Other stimuli that cause GLUT4 upregulation, such as 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside, also activate p38 and ERK5 (1, 14). Therefore, future studies should investigate the role of p38 and ERK5 in the caffeine-induced increase in GLUT4 expression.

In summary, we have shown that caffeine increases the contents of GLUT4 mRNA, reduces the content of HDAC5 in the nucleus, increases the acetylation of histone H3 at the MEF2 site on the *Glut4* gene, and increases the binding of MEF2A to the GLUT4 promoter in C₂C₁₂ myotubes. These effects of caffeine are reversed by dantrolene or KN93, indicating that caffeine affects GLUT4 transcription via Ca²⁺-dependent CaMK signaling.

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GRANTS

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