Class II Histone Deacetylases Downregulate *GLUT4* Transcription in Response to Increased cAMP Signaling in Cultured Adipocytes and Fasting Mice

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Insulin-mediated glucose uptake is highly sensitive to the levels of the facilitative glucose transporter protein, GLUT4. Repression of *GLUT4* expression is correlated with insulin resistance in adipose tissue. We have shown that differentiation-dependent *GLUT4* transcription was under control of class II histone deacetylases (HDACs). We hypothesized that HDACs may regulate gene expression in adipocytes as a result of adrenergic activation. To test this hypothesis, we activated cAMP signaling in 3T3-L1 adipocytes and in mice after an overnight fast. Chromatin immunoprecipitation experiments showed the association of HDAC4/5 with the *GLUT4* promoter in vivo and in vitro in response to elevated cAMP. Knockdown of HDACs by small interfering RNA in cultured adipocytes prevented the cAMP-dependent decrease in *GLUT4* transcription. HDAC4/5 recruitment to the *GLUT4* promoter was dependent on the *GLUT4* liver X receptor (LXR) binding site. Treatment of cells with an LXR agonist prevented the cAMP-dependent decrease in *GLUT4* transcription. A loss of function mutation in the LXR response element was required for cAMP-dependent downregulation of *GLUT4* expression in vitro, in fasted mice, and in mice subjected to diet-induced obesity. This suggests that activation of LXR signaling can prevent loss of *GLUT4* expression in diabetes and obesity. ***Diabetes*** **61:1404–1414, 2012**

Glucose homeostasis is partly regulated by the facilitative GLUT4, expressed in heart, skeletal muscle, and adipose tissue (1). GLUT4 expression changes in response to changing physiologic states such as fasting, obesity, and diabetes (2–6). Insulin-resistant glucose transport in adipose tissue results from decreased GLUT4 expression due largely to a decrease in *GLUT4* transcription (7,8). Previous work demonstrated that the *GLUT4* promoter is governed by at least three *cis*-acting elements: a myocyte enhancer factor 2 (MEF2) family domain that binds MEF2 transcription factors; domain I that binds a novel transcriptional factor, *GLUT4* enhancer factor (GEF); and the liver X receptor (LXR) response element (LXRE) that binds LXR-α in adipocytes (9,10). The transcription factors appear to create a docking platform that allows potential coactivators and corepressors to bind and regulate *GLUT4* expression, although we do not yet fully understand the coactivators and corepressors responsible for regulated expression of *GLUT4*.

We established a role for class II histone deacetylases (HDAC)s in the regulation of *GLUT4* expression (11,12). This work revealed that HDAC5 plays a central role in repression of *GLUT4* transcription in preadipocytes and that other class II HDACs, including HDAC4 and HDAC9, may have functional redundancy in conditions when HDAC5 is reduced. In the current study, we tested the hypothesis that *GLUT4* is regulated by class II HDACs in differentiated adipocytes as well as in preadipocytes. We demonstrated that HDAC4 and HDAC5 are both capable of specifically regulating the *GLUT4* promoter. Although HDAC5 is the predominant class II HDAC that binds the *GLUT4* promoter in preadipocytes, we found that HDAC4 was the predominant class II HDAC isoform bound to the *GLUT4* promoter under conditions where *GLUT4* transcription was downregulated in the adipocytes. Further, we demonstrate that adrenergic activation by isoproterenol downregulated *GLUT4* transcription by increasing class II HDAC association with the *GLUT4* promoter by a process requiring the GLUT4 LXRE.

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RESEARCH DESIGN AND METHODS

Cell culture and transfections. 3T3-L1 cells were maintained and transfected via electroporation as previously described (12). Cells were treated with a final concentration of 25 μmol/L forskolin (Calbiochem), 25 μmol/L isoproterenol hydrochloride (Sigma), or 0.1 μmol/L TO-901317 (Sigma).

Animals. C57BL/6 mice were used for all experiments. In some experiments, transgenic mice were used that carried a human *GLUT4* promoter/chloramphenicol acetyltransferase (CAT) reporter construct that is fully functional (895-hG4-CAT), and another line carrying a similar reporter with a loss of function mutation in the *GLUT4* LXRE, as previously described (10). All mice were kept on a 12-h light/dark cycle in a temperature- and humidity-controlled room with free access to water and standard chow. Food was removed at ~5 P.M. the night before when mice were challenged with fasting, but they had free access to water. The fasted animals were killed the following morning and tissues were isolated, flash-frozen, and stored for further analysis.

Obesity was induced by ad libitum feeding with a 60% lard diet (Research Diets) for 8 weeks. For these studies, nonobese control animals were maintained by ad libitum feeding with a 10% fat diet (Research Diets) for the same period. All procedures using animals were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

Small interfering RNA transfections. Cells in experiments using small interfering (si) RNA were transfected as previously described (12).

Chromatin immunoprecipitations. Chromatin immunoprecipitation (ChIP) reactions were performed as previously described (10) for cultured adipocytes with one modification: the α-LXR ChIP was performed with an additional crosslinking step using 2 mmol/L disuccinimidyl glutarate in 1× PBS and 1 mmol/L $MgCl_2$ for 45 min before crosslinking with 1% formaldehyde. For adipose tissues from fed or fasted mice, the following modifications were made: ~200 mg of adipose tissue was placed in Ham's F12 media containing 1% formaldehyde. Tissue was homogenized briefly with the Tissue Tearor and incubated at room temperature for 15 min, rocking end-over-end. The crosslinking was stopped with 0.125 mol/L glycine for 5 min. The nuclei were pelleted by centrifugation at 13,000 rpm for 15 min. The nuclear pellet was resuspended in high salt lysis buffer (Santa Cruz Biotechnology), sonicated, and treated the same as cultured cells for the rest of the procedure (10). DNA recovered from ChIP reaction was subjected to quantitative PCR (q-PCR) and analyzed as percent of input and normalized to nonimmune rabbit IgG (Cell Signaling). All q-PCR analysis were run using a CFX96 real-time (RT)-PCR detection system thermal cycler (Bio-Rad).

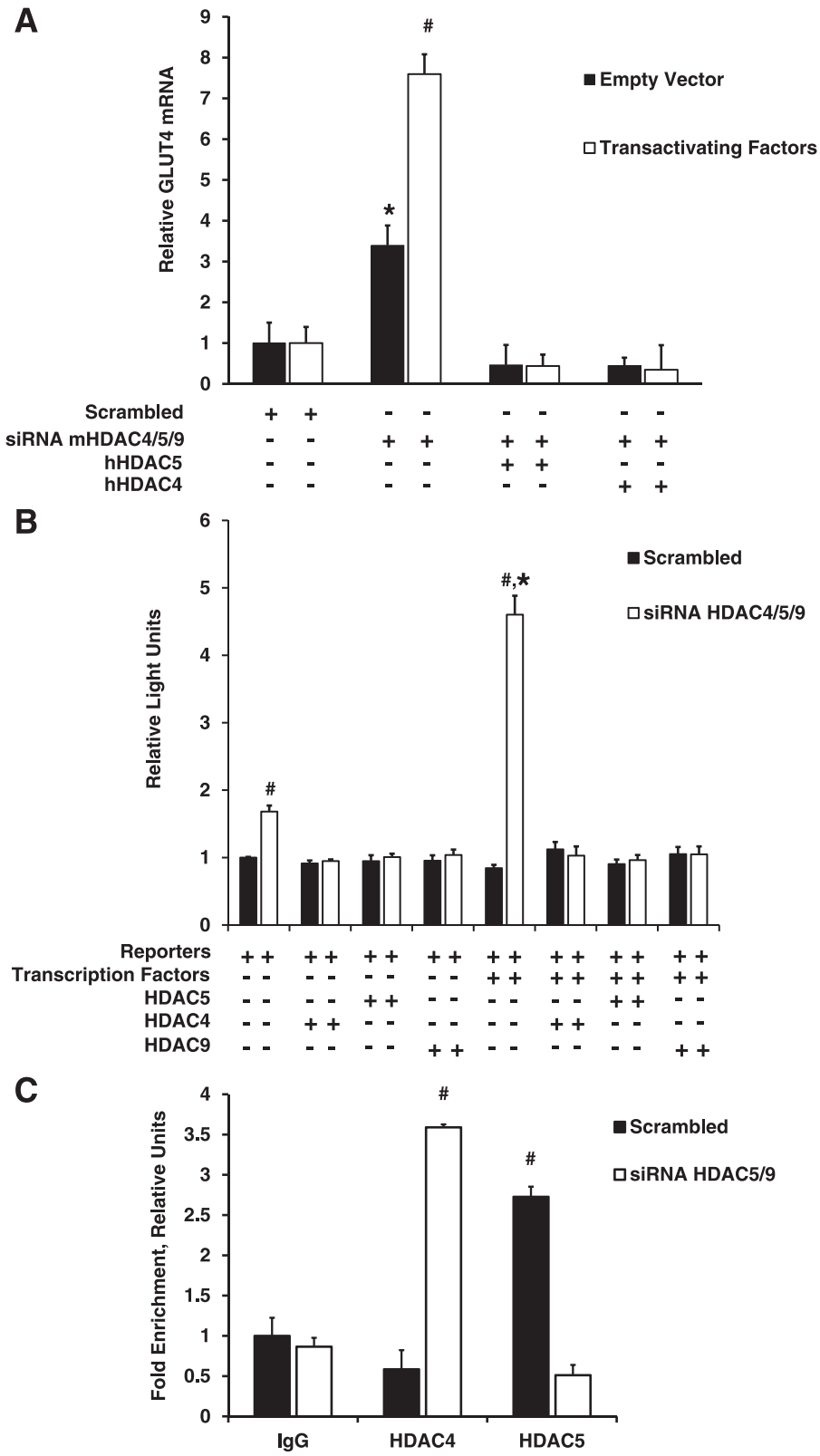

FIG. 1. Class II HDACs are functionally redundant, but HDAC5 is the primary *GLUT4* regulator in 3T3-L1 preadipocytes. Preadipocytes were transiently transfected with scrambled siRNA or HDAC4/5/9-specific siRNA, as indicated by +. The cells were used for the following experiments: *A*: After 3 days of siRNA treatment, cells were transiently transfected again with pcDNA3 (empty vector), MEF2A and GEF (transcription factors), or human HDAC4/human HDAC5, as indicated. Data are from at least three independent experiments and are expressed as mean and SEM and analyzed by one-way ANCOVA. *$P < 0.05$ (statistically significant) over scrambled siRNA, empty vector control. #$P < 0.05$ (statistically significant) over all other conditions. *B*: After 3 days of siRNA treatment, cells transiently transfected again with luciferase reporter plasmids -895-hGLUT4-luc (for promoter activity) and pRLTK (for transfection efficiency), with and without plasmids encoding GEF, MEF2A, wild-type HDAC4, and wild-type HDAC5. Mean data and SEM from three independent experiments done in duplicate are shown; data were analyzed by a one-way ANCOVA. #$P < 0.05$ (statistically significant) over scrambled siRNA, reporters alone. *$P < 0.05$ (statistically significant) over all other conditions. *C*: After 3 days of siRNA treatment, ChIP was performed using anti-HDAC4, anti-HDAC5, and nonimmune IgG

Immunoblot analysis. Samples were treated as previously described (12). Denatured samples were fractionated by SDS-PAGE using 10% polyacrylamide gels for cAMP-responsive element–binding (CREB) and phospho-CREB blots and 7.5% polyacrylamide gel for HDAC blots and transferred to polyvinylidene fluoride membranes. Membranes were probed with rabbit α-CREB (Cell Signaling), rabbit α-phospho–CREB (Cell Signaling), rabbit polyclonal α-HDAC5 antibodies (Millipore), or rabbit polyclonal α-HDAC4 (Cell Signaling) antibodies overnight at 4°C. After incubation with labeled secondary antibodies, membranes were quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

RNA extraction and q-RT-PCR. RNA was extracted and prepared as previously described (12). Primer sequences for *GLUT4* and *CAT* mRNA were as described (10). All q-RT-PCRs were run using a CFX96 RT-PCR detection system thermal cycler (Bio-Rad).

Luciferase assays. 3T3-L1 adipocytes were differentiated and electroporated as previously described (12). Luciferase assays were performed 24 h after transfection using the Dual-Glo luciferase kit (Promega).

Statistical analysis. Mean and standard error of the mean is reported. Differences were analyzed by a one-way ANCOVA or Student *t* test.

RESULTS

We previously established that multiple class II HDACs are capable of regulating the *GLUT4* promoter (12). Although we demonstrated that HDAC4, -5, and -9 each inhibited both *GLUT4* enhancer factor (GEF)– and myocyte-specific enhancer factor 2A (MEF2A)–dependent gene transcription in vitro, simultaneous siRNA-mediated knockdown of all three HDACs was required to mediate changes in *GLUT4* gene expression in vivo. These data suggest a possible redundant or compensatory function between the HDAC isoforms (12). To test this possibility, we over-expressed siRNA-resistant forms of HDAC4 and HDAC5 to rescue the inhibitory function in 3T3-L1 preadipocytes in which HDAC4, HDAC5, and HDAC9 were knocked down (Fig. 1*A*). As previously described, cells were transiently transfected with a combination of HDAC4-, HDAC5-, or HDAC9-specific siRNA (Ambion) or a nonspecific siRNA 72 h before the rescue (12). Cells were transfected again with either empty vector or transactivating factors (MEF2A and GEF) to activate the endogenous promoter, and plasmids encoding either human HDAC4 or HDAC5 as indicated (Fig. 1*A*). Endogenous *GLUT4* mRNA was inhibited by overexpressing each HDAC4 or HDAC5 individually, indicating that each of these class II HDACs can inhibit GLUT4 expression in preadipocytes.

Next, we used an in vitro transcription assay to quantify promoter activity after rescue of the class II HDAC knockdown (12). Preadipocytes were transiently transfected with scrambled or HDAC-specific siRNA for 72 h, as described above. After 72 h, cells were transfected a second time with the minimal functional human *GLUT4* promoter driving the expression of a firefly luciferase gene (-895-hGLUT4-luc), as previously characterized (11). Activity of the human *GLUT4* promoter in transient transfection assays is low compared with the activity of the promoterless reporter. To boost the signal, the reporter construct was cotransfected with MEF2A and GEF (11). To recover HDAC function, cells were cotransfected with human HDAC4, -5, or -9 as indicated (Fig. 1*B*). Transfection efficiency was accounted for by normalization to the control plasmid *Renilla* luciferase (pRLTK-luc), for transfection efficiency. As previously described, knockdown of the class II HDACs in preadipocytes, *GLUT4* expression increases significantly (Fig. 1*B*) (12). Rescue of the knockdown was achieved by overexpression of human HDAC4, -5, or- 9, suggesting redundant function. To begin determining which HDAC isoform(s) are physiologically relevant, we used a ChIP to determine if HDAC4 and/or HDAC5 (antibodies for HDAC9 are not available for ChIP) were bound to the *GLUT4* promoter in preadipocytes in vivo. When cells were treated with scrambled siRNA, we observed that HDAC5 bind the *GLUT4* promoter, whereas HDAC4 binding was no different than nonspecific IgG (Fig. 1*C*). This indicated that HDAC5 preferentially binds to the *GLUT4* promoter in a preadipocyte. Only when cells were treated with siRNA specific for HDAC5 and HDAC9 was there a significant increase in HDAC4 binding to the *GLUT4* promoter. Taken together, our data strongly support a model in which HDACs can be functionally redundant but do not directly compete for binding to the *GLUT4* promoter in preadipocytes.

A role for HDAC regulation of *GLUT4* expression in mature adipocytes has not been studied. We have previously shown that HDAC4 and HDAC5 are expressed in 3T3-L1 adipocytes but are largely excluded from the nuclei as a function of differentiation (11,12). This raised the possibility that HDAC4 or HDAC5 can be redistributed to the nucleus in 3T3-L1 adipocytes in response to specific intracellular signals. For example, cAMP has been implicated in HDAC5 redistribution into the nucleus from the cytosol in neurons (13). To test if this occurs in adipocytes, we elevated cAMP levels with 25 μmol/L forskolin. Forskolin-dependent cAMP signaling was confirmed by Western blotting analysis of phospho-CREB. Forskolin treatment increased phospho-CREB levels by 30 min, and they remained elevated for at least 6 h (Fig. 2*A*). We next tested the effect of forskolin treatment on HDAC localization. 3T3-L1 adipocytes were treated with 25 μmol/L forskolin for the indicated times. Nuclear extracts were prepared and subjected to Western blotting analysis for HDAC4 and HDAC5 (Fig. 2*B*). HDAC4 and HDAC5 nuclear content both increased as a result of forskolin treatment. To determine whether nuclear accumulation resulted in increased binding to the *GLUT4* promoter, ChIP was performed using antibodies against HDAC4 or HDAC5 (Fig. 2*C*). The ChIP experiments revealed that HDAC4 binding to the *GLUT4* promoter was preferentially increased as a result of forskolin treatment.

If forskolin-dependent changes in HDAC4 binding regulate *GLUT4* transcription, then forskolin treatment should decrease *GLUT4* mRNA and *GLUT4* transcriptional activity. To test this prediction, we measured *GLUT4* mRNA and found that forskolin treatment overnight resulted in a 60% reduction of *GLUT4* mRNA (Fig. 2*D*). Next, we assayed for transcriptional activity by transiently transfecting adipocytes using the -895-hGLUT4-luc reporter assay (11,12). The -895-hGLUT4-luc reporter was activated by cotransfection with MEF2A and GEF and treated without or with 25 μmol/L forskolin for the duration of the transfection period to ensure that cAMP was elevated while transcription of the reporter construct was occurring. As expected, cotransfection of -895-GLUT with MEF2A and GEF increased promoter activity sevenfold. Forskolin treatment resulted in a decrease (about fourfold) in transcriptional activity despite the presence of activating transcription factors (Fig. 2*E*).

antibodies. q-PCR was used to analyze the results. A ratio of expression to input was taken and normalized to control nonimmune IgG. Mean and SEM from three independent experiments is shown; data were analyzed by a one-way ANCOVA. #$P < 0.003$ (statistically significant) increase over background nonimmune IgG.

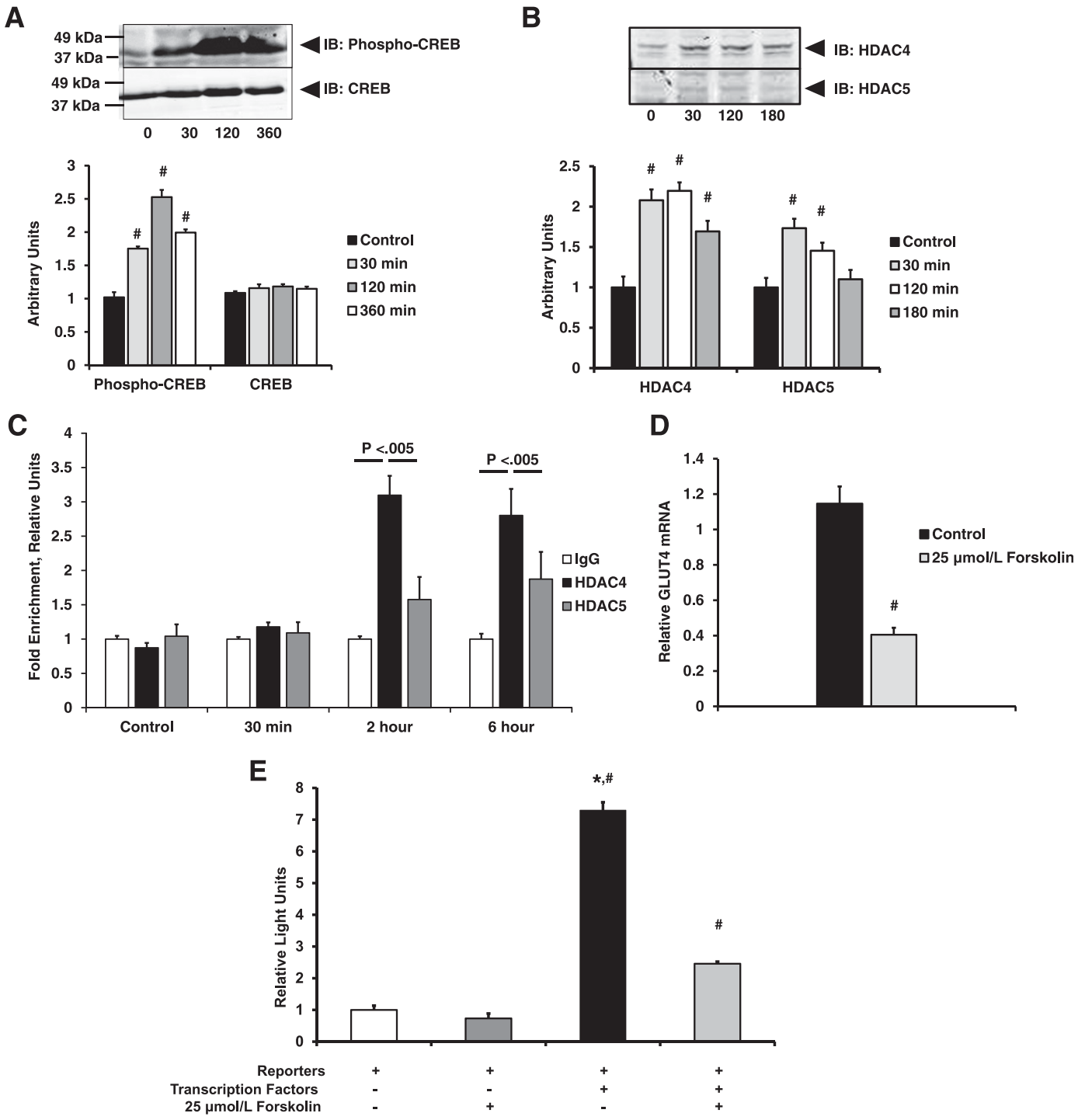

FIG. 2. Forskolin increased intracellular cAMP levels, induced HDAC4 nuclear localization, and downregulated the *GLUT4* promoter. *A*: SDS-PAGE was used to analyze 120 µg of nuclear extracts of 3T3-L1 adipocytes 6 days after differentiation treated with or without 25 µmol/L forskolin, which were immunoblotted (IB) for endogenous CREB and phospho-CREB, as indicated. Densitometry was analyzed by Li-Cor Imagining software and quantification represents SEM of three independent experiments. #$P < 0.05$ (statistically significant) over untreated control extracts. *B*: SDS-PAGE was used to analyze 120 µg of nuclear extracts from 3T3-L1 adipocytes 6 days after differentiation, treated with or without 25 µmol/L forskolin, which were immunoblotted for endogenous HDAC4 or HDAC5, as indicated. Densitometry was analyzed by infrared spectroscopy and quantification represents mean and SEM of three independent experiments. #$P < 0.05$ (statistically significant) over untreated control extracts. *C*: 3T3-L1 adipocytes 6 days after differentiation were treated or untreated with 25 µmol/L forskolin for the indicated time points, and ChIP was performed using the indicated anti-HDAC4, anti-HDAC5, and anti-nonimmune IgG antibodies. Results were analyzed by q-PCR and determined by an expression-to-input ratio and normalized to nonimmune IgG controls. Data are the mean and SEM from three independent experiments analyzed by one-way ANCOVA ($P < 0.005$ is statistically significant). *D*: 3T3-L1 adipocytes 6 days after differentiation were treated or untreated with 25 µmol/L forskolin for 3 h and isolated RNA was subjected to q-RT-PCR to determine relative *GLUT4* mRNA. #$P < 0.003$ (statistically significant). *E*: 3T3-L1 adipocytes 6 days after differentiation were transiently transfected with -895-hGLUT4-luc (for promoter activity) and pRLTK (for transfection efficiency), with and without plasmids encoding GEF and MEF2A (transcription factors). Cells were treated or untreated with 25 µmol/L forskolin throughout the 18-h transfection. Data are the mean and SEM from three independent experiments analyzed by a one-way ANCOVA. #$P < 0.05$ (statistically significant) compared with reporters alone. *$P < 0.05$ significant compared with all other conditions.

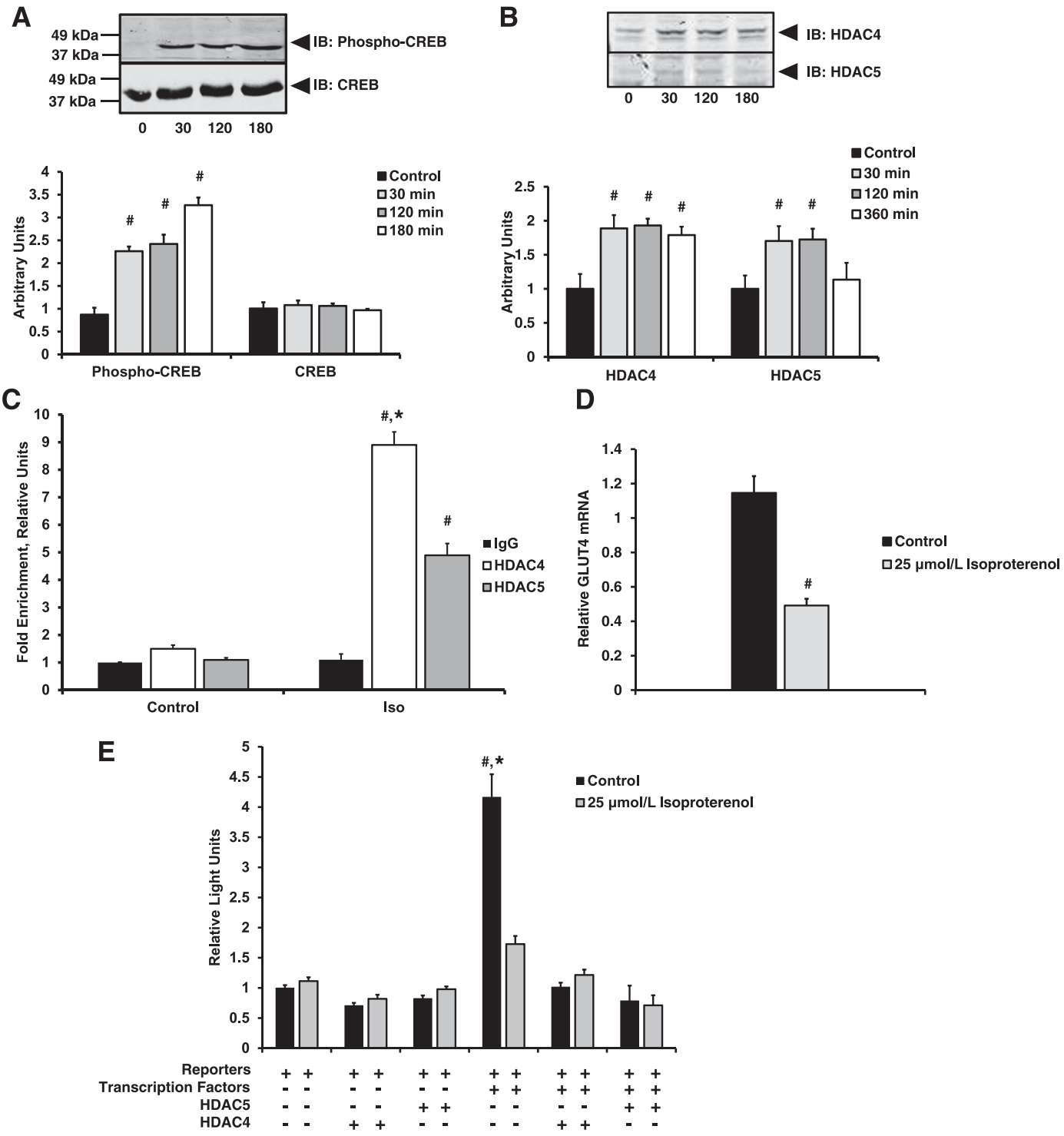

FIG. 3. Isoproterenol treatment increased intracellular cAMP levels, induced HDAC4 nuclear localization and down-regulated the *GLUT4* promoter. *A*: A total of 120 μg of nuclear extracts of 3T3-L1 adipocytes 6 days after differentiation treated with or without 25 μmol/L isoproterenol were analyzed by SDS-PAGE and immunoblotted for endogenous CREB and phospho-CREB, as indicated. Densitometry analysis was quantified using Li-Cor Imagining software. Date represent mean and SEM of three independent experiments. #Statistical significance vs. untreated control extracts. *B*: A total of 120 μg of nuclear extracts of 3T3-L1 adipocytes 6 days after differentiation treated with or without 25 μmol/L isoproterenol were analyzed by SDS-PAGE and immunoblotted for endogenous HDAC4 or HDAC5 as indicated. Densitometry analysis was quantified using Li-Cor Imagining software. Data represent mean and SEM of three independent experiments. #Statistical significance vs. untreated control extracts. *C*: 3T3-L1 adipocytes 6 days after differentiation were treated or untreated with 25 μmol/L isoproterenol (Iso) for 3 h, and ChIP was performed using the indicated anti-HDAC4, anti-HDAC5, and anti-nonimmune IgG antibodies. Results were analyzed by q-PCR and determined by an expression-to-input ratio and normalized to nonimmune IgG controls. Data represent mean and SEM from three independent experiments analyzed by one-way ANCOVA. #,*$P < 0.05$ (statistically significant) for controls compared with all other conditions. *D*: 3T3-L1 adipocytes 6 days after differentiation were treated or untreated with 25 μmol/L isoproterenol for 3 h, and isolated RNA was subjected to q-RT-PCR to determine relative *GLUT4* mRNA. #$P < 0.003$. *E*: 3T3-L1 adipocytes 6 days after differentiation were transiently transfected with luciferase reporter plasmids -895-hG4-luc (for promoter activity) and pRLTK (for transfection efficiency), with and without plasmids encoding GEF and MEF2A (transcription factors). Cells were treated or untreated with 25 μmol/L isoproterenol, as indicated. Data represent mean and SEM of three independent experiments analyzed by a one-way ANCOVA. #$P < 0.05$ vs. reporters alone. *$P < 0.05$ vs. all other conditions.

To activate cAMP using a more physiologic mechanism, we used isoproterenol, a β-adrenergic receptor agonist, to initiate cAMP signaling. We performed a time-course to measure phospho-CREB. Similar to forskolin, isoproterenol increased phospho-CREB by 30 min of treatment, and it remained elevated for at least 3 h (Fig. 3*A*). Isoproterenol treatment increased nuclear accumulation of both HDAC4 and HDAC5 (Fig. 3*B*). We next performed ChIP assays to determine if HDAC4 and HDAC5 binding to the *GLUT4* promoter also increased as a result of isoproterenol treatment. The ChIP assay revealed that the association with the *GLUT4* promoter increased in both HDAC4 and HDAC5, with HDAC4 increasing more than HDAC5 (Fig. 3*C*).

Similar to treatment with forskolin, overnight treatment with isoproterenol reduced *GLUT4* by ~50% (Fig. 3*D*). To confirm that this is a transcriptional effect, we tested isoproterenol to regulate expression of the -895-hGLUT4-luc transcriptional report. Overnight treatment with isoproterenol inhibited GEF- and MEF2A-dependent activation of -895-hGLUT4-luc (Fig. 3*E*). Cotransfection with plasmids encoding HDAC4 or HDAC5 inhibited transcriptional activation of -895-hGLUT4-luc, with no further potentiation of the effect when isoproterenol was added (Fig. 3*E*).

To determine if isoproterenol-dependent changes in HDAC association with the GLUT4 reporter are responsible for the changes in *GLUT4* transcription, we used siRNA knockdown of HDAC to determine if HDAC protein expression was required for this effect. To test this, preadipocytes were transiently transfected with scrambled siRNA or HDAC-specific siRNA 72 h before the assay under conditions (i.e., knockdown of HDAC4/5/9) previously shown to increase GLUT4 mRNA in preadipocytes (12). After 72 h, cells were transfected a second time with the -895-hGLUT4-luc plasmid to assay for *GLUT4* transcriptional activity. The -895-hGLUT4-luc was transactivated with plasmids encoding MEF2A and GEF and treated without or with 25 μmol/L forskolin or 25 μmol/L isoproterenol as indicated (Fig. 4). We observed that forskolin- and isoproterenol-dependent inhibition was abolished when HDACs were knocked down. Our data indicate that HDAC proteins must be present for either compound to inhibit *GLUT4* transcription. Taken together, these data support our hypothesis that forskolin and isoproterenol treatment alters *GLUT4* expression through HDAC association with the *GLUT4* promoter, likely through a cAMP-dependent signaling pathway.

Using forskolin and isoproterenol treatment, we have shown that HDAC4 and HDAC5 associate with the *GLUT4* promoter to reduce the rate of transcription. We next determined if HDAC4 and HDAC5 associate with the *GLUT4* promoter in vivo when *GLUT4* mRNA is downregulated. To test this, we subjected mice to an overnight fast, a condition known to decrease *GLUT4* gene expression (2,4,6,14–16). Mice were allowed to have free access to food (fed) or were fasted overnight and killed. White adipose tissue and total proteins were extracted for Western blotting analysis of phospho-CREB (Fig. 5*A*). Mice fasted overnight demonstrated a significant increase in phospho-CREB levels compared with fed control mice, while not displaying any significant changes in total CREB, consistent with elevated cAMP signaling. Overnight fasting reduced *GLUT4* mRNA in perigonadal white adipose tissue by 50% (Fig. 5*B*), which is consistent with previous literature (8,16–18).

Next, we determined if the changes in *GLUT4* mRNA expression under the fasting protocol correlated with changes in class II HDACs association with the *GLUT4* promoter. Nuclei were prepared from adipose tissue of mice that were fasted overnight or were allowed free access to food. We did not observe a difference between nonspecific IgG and HDAC4 or HDAC5 binding to the *GLUT4* promoter in animals that were allowed access to food overnight (Fig. 5*C*). However, when animals were challenged with overnight fasting, we observed a large increase of both HDAC4 and HDAC5 binding to the *GLUT4* promoter. Similar to results observed in 3T3-L1 adipocytes challenged with either forskolin or isoproterenol, HDAC4 binding was higher than HDAC5.

Previous studies have demonstrated that the nuclear orphan receptor LXR activation is linked to HDAC-mediated transcriptional repression (19). Work from our laboratory has demonstrated a role for LXR in *GLUT4* transcriptional regulation (10,20). Therefore, we tested the role of LXR signaling on isoproterenol-mediated inhibition of the

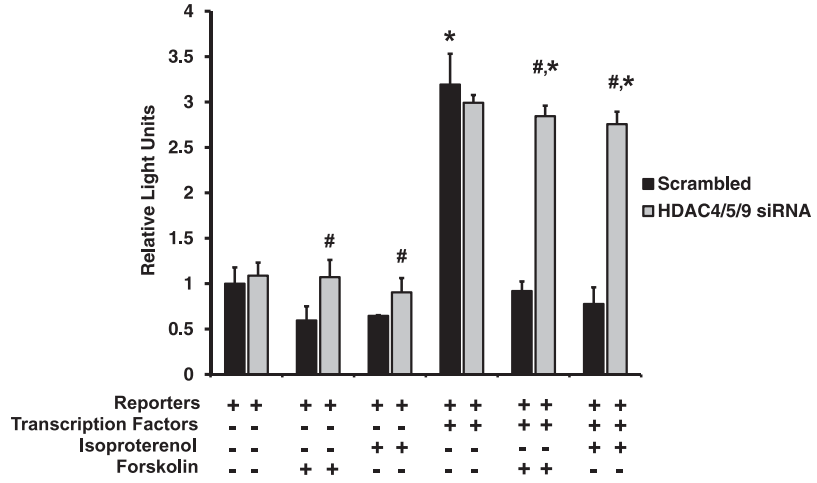

FIG. 4. siRNA-mediated knockdown of class II HDACs abolish the isoproterenol effect on *GLUT4* inhibition. 3T3-L1 preadipocytes were transiently transfected with scrambled siRNA or HDAC4/5/9-specific siRNA and incubated for 3 days, then transfected a second time with luciferase reporter constructs -895-hGLUT4-luc (for promoter activity) and pRLTK (for transfection efficiency), with and without plasmids encoding GEF and MEF2A, and treated or untreated with forskolin or isoproterenol (+ indicates inclusion in transfection). Data represent mean and SEM from three independent experiments analyzed by a one-way ANCOVA. #Statistical significance ($P < 0.05$) vs. the respective scrambled siRNA condition. *$P > 0.003$ vs. other conditions.

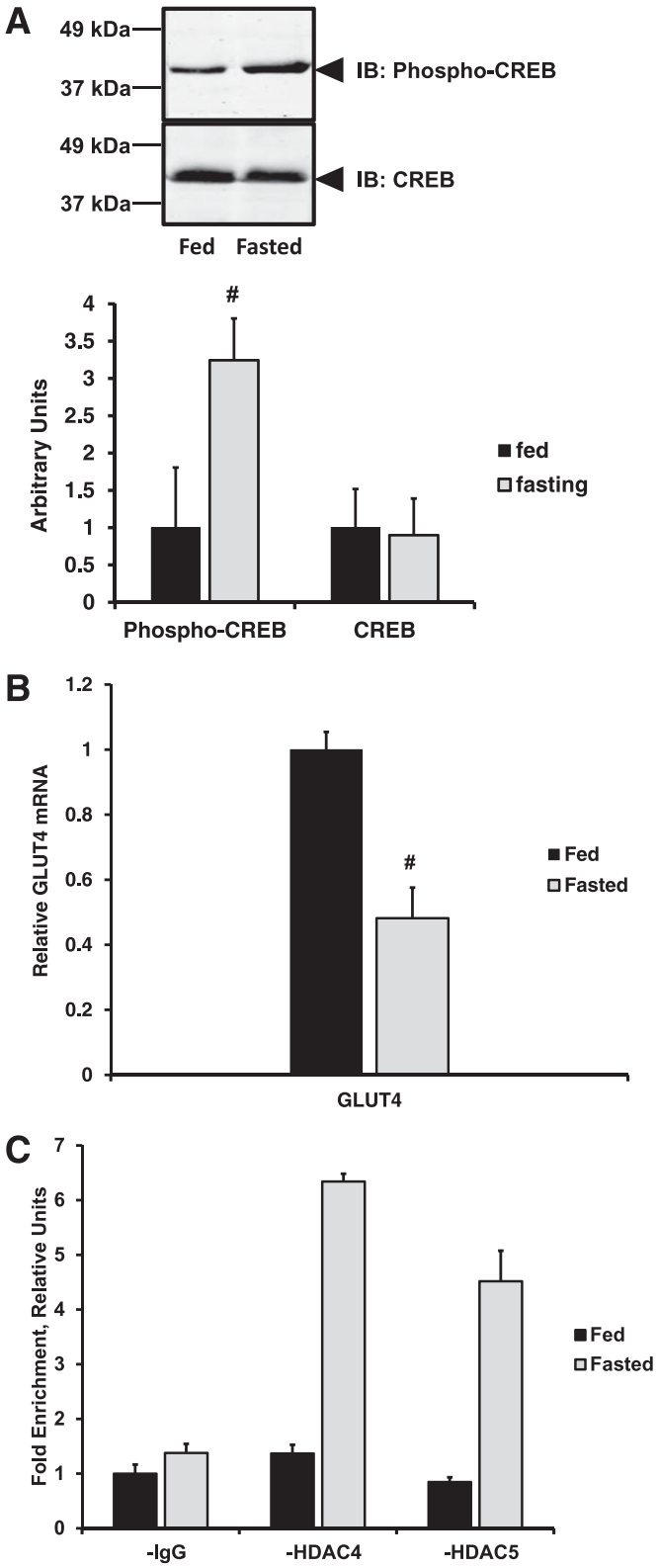

FIG. 5. Fasting mice exhibit elevated cAMP and increased enrichment of HDAC4 on the *GLUT4* promoter. *A*: C57BL/6 mice were allowed free access to food or fasted overnight and white adipose tissue (WAT) was collected. A whole-cell lysate was made from the WAT and analyzed by SDS-PAGE and immunoblotted (IB) for endogenous CREB and p-CREB, as indicated. Densitometry was analyzed by Li-Cor Imagining software, and quantification represents the mean and SEM of three independent experiments. #Statistical significance vs. extracts from fed controls. *B*: WAT from fed or fasting mice was collected, and q-RT-PCR of isolated RNA was used to determine relative *GLUT4* mRNA. #$P < 0.003$. *C*: C57BL/6 mice were allowed free access to food or fasted overnight and WAT was collected. ChIP analysis on the WAT was performed using the indicated anti-HDAC4, anti-HDAC5, and anti-nonimmune IgG antibodies. Results were analyzed by qPCR and determined by an expression-to-input ratio and normalized to nonimmune IgG controls. Mean and SEM from three independent experiments is shown and was analyzed by one-way ANCOVA. #$P < 0.001$ vs. nonimmune IgG control.

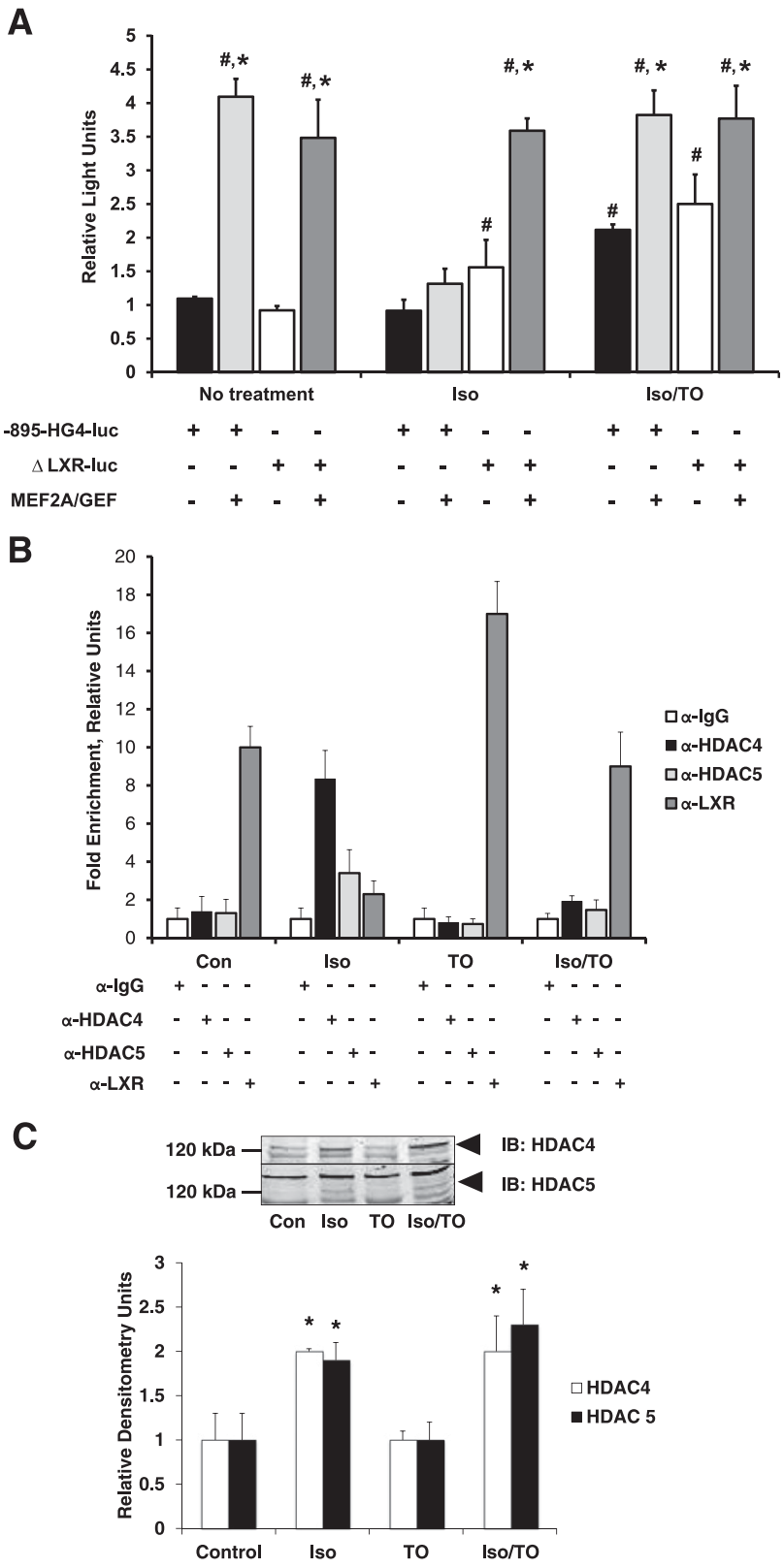

FIG. 6. The LXR agonist TO901317 rescues the inhibitory isoproterenol effect by dislodging HDAC4 from the *GLUT4* promoter. *A*: 3T3-L1 adipocytes 6 days after differentiation were transiently transfected with luciferase reporter plasmids -895-hGLUT4-luc or ΔLXR-hGLUT4-luc and pRLTK (for transfection efficiency), with and without plasmids encoding GEF and MEF2A (transcription factors). Cells were treated or untreated with 25 μmol/L isoproterenol (Iso) and/or 0.1 μmol/L TO901317 (TO), as indicated. Mean and SEM from three independent experiments is shown; data were analyzed by a one-way ANCOVA. #$P < 0.05$ (statistically significant) vs. with reporters alone. *Significant vs. all other conditions. *B*: 3T3-L1 adipocytes 6 days after differentiation were treated or untreated with 25 μmol/L isoproterenol and/or 0.1 μmol/L TO901317 for 3 h, and ChIP was performed using the indicated anti-HDAC4, anti-HDAC5, anti-LXR, and anti-nonimmune IgG antibodies. Results were analyzed by q-PCR and determined by an expression-to-input ratio and normalized to nonimmune IgG controls (Con). Mean and SEM from three independent experiments is shown and was analyzed by one-way ANCOVA. #$P < 0.001$ compared with nonimmune IgG control; *Significant compared with all

GLUT4 promoter. Toward this end, we transiently transfected adipocytes with the -895-hGLUT4-luc or Δ-LXR-hGLUT4-luc construct, as indicated (Fig. 6*A*). The Δ-LXR-hGLUT4-luc construct has a loss of function mutation in the LXRE sites on the *GLUT4* promoter, as previously characterized (10). The luciferase constructs were activated with transcription factors (MEF2A and GEF) and treated with isoproterenol alone or with TO901317 together (Fig. 6*A*). We observed a significant increase in activity when both constructs were activated with transcription factors. Cells treated with isoproterenol alone displayed a statistically significant decrease in *GLUT4* transcriptional activity of the -895-hGLUT4-luc. However, the Δ-LXR-hGLUT4-luc was not affected by isoproterenol treatment, indicating that LXR plays a key role in cAMP-dependent inhibition of the *GLUT4* promoter. To confirm the role of LXR, we treated the cells with the LXR agonist TO901317 and with isoproterenol. Interestingly, *GLUT4* transcriptional activity was restored, despite the presence of isoproterenol. The LXR agonist, TO901317, was able to overcome the inhibitory effect of isoproterenol-dependent regulation of *GLUT4* mRNA.

Because TO901317 relieved the inhibition of *GLUT4* transcription mediated by isoproterenol, we next determined if TO901317 changed HDAC binding to the *GLUT4* promoter. We treated the cells with TO901317 or isoproterenol, or both, and performed ChIP analysis. We observed a large increase in HDAC4 and HDAC5 binding to the *GLUT4* promoter in response to isoproterenol (Fig. 6*B*). The addition of TO901317 prevented the isoproterenol-dependent increase in HDAC binding to the *GLUT4* promoter (Fig. 6*B*). In contrast to HDACs, LXR binding to the LXRE was reduced by isoproterenol treatment and returned to normal when treated with TO901317 (Fig. 6*B*). TO901317 did not affect the accumulation of HDAC4 or HDAC5 in the nucleus (Fig. 6*C*). TO901317 treatment had no effect on the induction of phospho-CREB (data not shown).

Taken together, we have demonstrated that elevated cAMP levels can downregulate the *GLUT4* promoter by inducing HDAC4 binding to the *GLUT4* promoter through a mechanism that requires LXRE but can be inhibited by treatment with TO9001317. These data suggest that the regulated expression of *GLUT4* during the transition from fasting to feeding is regulated by cAMP signaling through the LXRE. To confirm that LXRE plays a physiologic role in modulation of *GLUT4* mRNA, we tested the requirement for the *GLUT4* LXRE in two models, fasting and diet-induced obesity, that are accompanied by changes in *GLUT4* expression. To estimate *GLUT4* transcription in the mouse model, we used transgenic mice engineered to carry a fully functional *GLUT4* promoter/reporter construct (-895-HG4-CAT) or a construct with a loss of function mutation in the *GLUT4* LXRE (Δ-LXR-CAT) (Fig. 7*A*) (10). Animals were fed a control diet (10% fat) or a high-fat diet (60% fat) for 8 weeks. The control-diet animals were deprived of food overnight or were fed ad libitum until they were killed (serving as the control for both experimental groups). *CAT* and endogenous *GLUT4* mRNA levels were quantified from perigonadal adipose tissue. As expected, endogenous *GLUT4* mRNA was decreased in perigonadal adipose tissue in response to both fasting and diet-induced obesity. Transgenic mRNA levels under the control of the -895-HG4 promoter changed in parallel with endogenous *GLUT4* (Fig. 7*B*). In contrast, the loss of function mutation in the *GLUT4* LXRE was unresponsive to fasting or diet-induced obesity, and transgenic mRNA levels were unchanged (Fig. 7*B*).

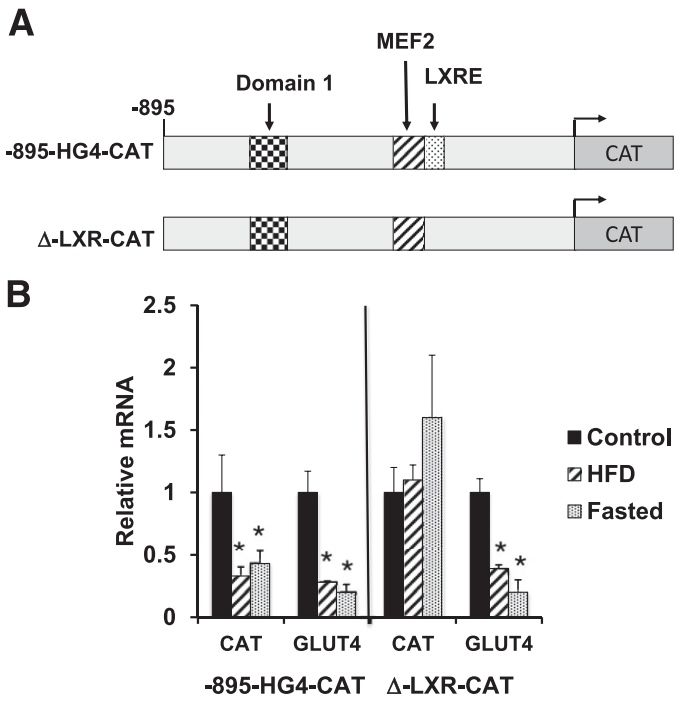

FIG. 7. *GLUT4* LXRE is required for downregulation of adipose *GLUT4* mRNA during fasting and diet-induced obesity. C57BL/6 mice (2- to 3-month-old) mice were fed a 10% fat diet (control) or 60% high-fat diet (HFD) for 8 weeks. Fasted animals were animals fed the 10% fat diet that were deprived of food. *A*: Schematic drawing depicts the constructs used to generate transgenic animals. *B*: Total mRNA from perigonadal adipose tissue was isolated and used to determine mRNA levels of *CAT*, *GLUT4*, and *actin*. *CAT* and *GLUT4* mRNA levels were normalized to actin, and the normalized mRNA levels were expressed as percent of the control mRNA for each experimental group. Data were analyzed using a two-tailed Student *t* test. *$P < 0.05$ for difference between experimental and control groups.

DISCUSSION

GLUT4 gene expression in adipose tissue is sensitive to changes in metabolic state, including the fasting and diabetes (8,18,21). Understanding how the *GLUT4* gene promoter responds to changes in metabolism is a key to understanding the molecular events that underlie this important adaptation to changing physiology. Using transgenic mice to elucidate the key regulatory domains present in the *GLUT4* promoter, we determined the MEF2 binding domain and domain 1 were both required for *GLUT4* gene transcription (9,22).

We demonstrated that the MEF2 and GEF transcription factors work synergistically to transactivate the *GLUT4* promoter and that both are necessary but not sufficient to drive *GLUT4* transcription (9,22). These elements, when ligand bound, produce a scaffold that allows the binding of coactivators and corepressors to modulate the *GLUT4*

other conditions. *C*: Nuclear extracts (60 μg) from 3T3-L1 adipocytes 6 days after differentiation treated without or with 25 μmol/L isoproterenol, 0.1 μmol/L TO901317, or 25 μmol/L isoproterenol and 0.1 μmol/L TO901317, were analyzed by SDS-PAGE and immunoblotted (IB) for endogenous HDAC4 or HDAC5, as indicated. Densitometry was analyzed by infrared spectroscopy and quantification represents mean and SEM of three independent experiments. *$P < 0.05$ vs. untreated control extracts.

promoter in response to altered metabolic states. Among the potential corepressors that bind this scaffold are the class II HDACs (11,12).

In this report, we provide several lines of evidence supporting the notion that HDACs can be functionally redundant with regard to *GLUT4* transcription but have isoform-specific roles based on the stage of differentiation. We, along with others, have observed that class II HDACs potentially compensate for each other, resulting in the requirement to knockdown multiple HDACs to achieve a phenotype (12,23,24). Therefore, we sought to determine the conditions under which HDACs compensate for each other. We demonstrated that class II HDACs, specifically HDAC4 and 5, are functionally redundant in regards to the *GLUT4* promoter in preadipocytes; however, the HDAC5 preferentially binds the *GLUT4* promoter in preadipocytes. HDAC4 only binds to the *GLUT4* promoter in the preadipocyte stage when HDAC5 is knocked down. Therefore, despite the structural similarities, HDAC4 does not compete with HDAC5 to regulate the *GLUT4* promoter in preadipocytes. Rather, HDAC4 can only bind the *GLUT4* promoter in preadipocytes when HDAC5 and HDAC9 are depleted. The specific contribution of HDAC9 is not yet clarified because suitable antibodies for ChIP and Western blotting analysis were not available.

The role of HDAC proteins in regulation of *GLUT4* in differentiated adipocytes is different from preadipocytes. Early work on *GLUT4* transcriptional regulation demonstrated a link between elevated cAMP levels and decreased *GLUT4* expression (17,18). Recent work showed that class II HDACs changed their localization pattern in response to elevated cAMP; therefore, we reasoned that class II HDACs regulate the *GLUT4* promoter in differentiated adipocytes via activation of cAMP. To this end, we determined that HDAC4 and HDAC5 increased their association with the *GLUT4* promoter in response to stimuli (forskolin or isoproterenol) that increased intracellular cAMP. Unlike preadipocytes, HDAC4 and HDAC5 binding to the promoter both increased in circumstances in differentiated adipocytes. Thus, there appears to be a clear, distinct role for both HDAC4 and 5 at different developmental stages.

To ensure that isoproterenol and forskolin were both working through class II HDACs, we used HDAC-specific siRNA knockdown in conjunction with the treatments in our luciferase assay (Fig. 4). This effectiveness of the siRNA-mediated knockdown of HDACs has been previously characterized (12), and the effect of both treatments on cAMP has also been demonstrated (Fig. 2 and 3). By knocking down HDACs and abolishing the inhibitory effect of either treatment, we firmly established the role of HDACs in cAMP-mediated *GLUT4* repression.

Although our in vitro data are clear, we needed to establish the physiologic relevance of the finding. Isoproterenol activates a counter-regulatory hormone response, which would be activated in the fasted state, a condition that leads to downregulation of *GLUT4* expression. We subjected mice to an overnight fast to test the effects on cAMP activation, *GLUT4* mRNA, and HDAC association with the *GLUT4* promoter. As expected, fasting increased cAMP signaling and decreased *GLUT4* mRNA. More importan, we found that HDAC4 and HDAC5 binding to the *GLUT4* promoter increased in vivo, similarly to what we observed in vitro (compare Figs. 2*C*, 3*C*, and 5*C*).

In this report, we have demonstrated that elevated cAMP negatively regulates the *GLUT4* promoter via HDACs. How cAMP signals HDAC redistribution in adipocytes to the nucleus is unclear. However, during times of metabolic stress like fasting, the need for glucose storage in adipose tissue is suppressed while enzymes regulating fatty acid release are activated, a function consistent with LXR signaling. The *GLUT4* promoter contains an LXRE binding domain, just eight base pairs downstream of the MEF2 binding domain. Our laboratory previously established a role for LXR in *GLUT4* regulation in response to inhibiting β-oxidation (10). We have now determined that cAMP signaling, fasting, and adaptation to obesity require the intact LXRE to specifically downregulate *GLUT4* gene transcription (Figs. 6 and 7). We observed that treatment of cells with TO901317, an LXR agonist, completely reversed the effects of isoproterenol on *GLUT4* promoter activity and HDAC4 and HDAC5 association with the *GLUT4* promoter. TO901317 treatment did not reverse the nuclear accumulation of HDAC4 or HDAC5 in isoproterenol-treated cells (Fig. 6*C*); however, it did reverse the isoproterenol-dependent loss in the LXR association with the *GLUT4* promoter (Fig. 6*B*). This suggests that the cAMP-dependent increases in HDAC association with the *GLUT4* promoter are mitigated by the LXRE ligands.

A recent study demonstrated that HDACs can bind and downregulate LXR-dependent gene transcription through a small ubiquitin-like modifier (SUMO)ylation of LXR (19). We have shown that treatment with TO901317 increases the LXR-α association with the *GLUT4* promoter (Fig. 6*B* [10]); therefore, cAMP is not likely to be acting through the SUMOlyation pathway. The LXRE is clearly required for downregulation of *GLUT4* mRNA in vitro and in vivo given that the loss of function mutation even though it no longer supports downregulation of the gene. This suggests that when LXR binding to the LXRE is decreased, another ligand may bind and that this ligand may facilitate HDAC association with the *GLUT4* promoter. This model will be tested in future studies in our laboratory.

In summary, we have demonstrated that despite the functional redundancy of HDACs, HDAC4 and HDAC5 have physiologically distinct functions at different stages of adipocyte differentiation to regulate the *GLUT4* promoter. Furthermore, we have demonstrated that cAMP-dependent downregulation of *GLUT4* mRNA expression in vivo is dependent on nuclear localization of HDAC4 and HDAC5 and the presence of a functional LXR binding element in the *GLUT4* promoter. This offers a new understanding of how cAMP-signaling pathways alter gene expression in adipose tissue.

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J.C.W. researched data and wrote the manuscript. B.A.G. researched data. A.L.O. researched data and reviewed and edited the manuscript. A.L.O. is the guarantor of this work and, as such, had full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

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