Acute Inhibition of Fatty Acid Import Inhibits GLUT4 Transcription in Adipose Tissue, but Not Skeletal or Cardiac Muscle Tissue, Partly Through Liver X Receptor (LXR) Signaling

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OBJECTIVE—Insulin-mediated glucose uptake is highly sensitive to the levels of the facilitative GLUT protein GLUT4. Transcription of the *GLUT4* gene is repressed in states of insulin deficiency and insulin resistance and can be induced by states of enhanced energy output, such as exercise. The cellular signals that regulate *GLUT4* transcription are not well understood. We hypothesized that changes in energy substrate flux regulate *GLUT4* transcription.

RESEARCH DESIGN AND METHODS—To test this hypothesis, we used transgenic mice in which expression of the chloramphenicol acetyltransferase (CAT) gene is driven by a functional 895-bp fragment of the human *GLUT4* promoter, thereby acting as a reporter for transcriptional activity. Mice were treated with a single dose of etomoxir, which inhibits the transport of long-chain fatty acids into mitochondria and increases basal, but not insulin-mediated, glucose flux. *GLUT4* and transgenic *CAT* mRNA were measured.

RESULTS—Etomoxir treatment significantly reduced *CAT* and *GLUT4* mRNA transcription in adipose tissue, but did not change transcription in heart and skeletal muscle. Downregulation of *GLUT4* transcription was cell autonomous, since etomoxir treatment of 3T3-L1 adipocytes resulted in a similar downregulation of *GLUT4* mRNA. *GLUT4* transcriptional downregulation required the putative liver X receptor (LXR) binding site in the human GLUT4 gene promoter in adipose tissue and 3T3-L1 adipocytes. Treatment of 3T3-L1 adipocytes with the LXR agonist, TO901317, partially restored *GLUT4* expression in etomoxir-treated cells.

CONCLUSIONS—Our data suggest that long-chain fatty acid import into mitochondria in adipose tissue may produce ligands that regulate expression of metabolic genes. ***Diabetes*** **59: 800–807, 2010**

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GLUT4 is the major insulin-responsive GLUT responsible for insulin-dependent glucose disposal. Insulin-mediated glucose homeostasis is exquisitely sensitive to the level of GLUT4 protein. Mice that express exogenous *GLUT4* gene in the major *GLUT4*-expressing tissues, or in skeletal muscle or adipose tissue alone, displayed enhanced insulin sensitivity and peripheral glucose utilization (1–4), whereas genetic ablation of one or both alleles of the GLUT4 gene result in insulin-resistant glucose disposal (5,6).

Expression of *GLUT4* mRNA is subject to tissue-specific, hormonal, and/or metabolic regulation. *GLUT4* mRNA expression is largely restricted to brown adipose, white adipose, skeletal muscle, and cardiac muscle tissues. Changes in *GLUT4* gene expression are observed in pathophysiological states of altered glucose homeostasis. In general, *GLUT4* mRNA expression is reduced in states of relative insulin deficiency such as streptozotocin-induced diabetes and chronic fasting (rev. in 7,8). Interestingly, the regulated steady-state levels of *GLUT4* mRNA differ in various GLUT4-expressing tissues. For example, streptozotocin-dependent changes in *GLUT4* mRNA expression occur much more rapidly in adipose tissue than skeletal muscle (9). Chronic fasting markedly reduces *GLUT4* mRNA levels in adipose tissue, but has little or no effect on *GLUT4* mRNA in skeletal muscle (10). Changes in steady-state levels of GLUT4 mRNA most likely reflect changes in the rate of mRNA synthesis (gene transcription) as opposed to changes in mRNA half-life, as evidenced by nuclear run-on assays showing that transcription is decreased in both adipose tissue and skeletal muscle in streptozotocin-induced diabetic animals (11,12), while the rate of the *GLUT4* gene transcription in skeletal muscle of fasted animals is increased (12).

Regulation of *GLUT4* gene expression within a narrow range (two to threefold) has a significant effect on whole body glucose homeostasis, suggesting that a fine control of GLUT4 levels is important for adaptation to extreme physiologic states such as prolonged fasting or to pathologic states such as chronic over nutrition. Because this gene is under complex metabolic control, the molecular basis for regulation of *GLUT4* gene expression in altered physiologic states has been difficult to solve.

We hypothesize that changes in substrate flux may importantly contribute to the regulation of *GLUT4* expression. These adaptive changes may emanate directly from specific substrate utilization or may result from hormonal

or neuronal signals that respond to changes in fuel sources or fuel needs.

In this article, transgenic mice carrying the chloramphenicol acetyltransferase (*CAT*) gene under the control of 895 bp of human *GLUT4* promoter (hGLUT4-CAT) were treated with etomoxir, an irreversible inhibitor of carnitine palmitoyl transferase (CPT)-1. This treatment acutely inhibits mitochondrial long-chain fatty acid (LCFA) import and oxidation, increases glucose oxidation in tissues that normally oxidize fatty acids, and lowers circulating levels of glucose (13–15). Etomoxir treatment caused decreased *GLUT4* transcription in adipose tissue but not in the heart or hindquarter skeletal muscle. The effect on *GLUT4* expression in adipose tissue was recapitulated in 3T3-L1 adipocytes and was mediated in part through a liver X receptor element (LXRE) site in the *GLUT4* promoter. Our data support the hypothesis that *GLUT4* expression is regulated by cell-autonomous tissue-specific metabolic signals.

RESEARCH DESIGN AND METHODS

Animals. Mice carrying a randomly integrated transgenic construct of the CAT gene driven by 895 bp of the human *GLUT4* promoter DNA (895-hGLUT4-CAT) were obtained as previously described (16). A loss of function mutation in the putative LXRE site of the human GLUT4 promoter was engineered into the 895-HG4-CAT fragment and renamed Δ-LXR-CAT. The mutation was identical to that reported by Dalen et al. (17), changing human *GLUT4* LXRE (shown in bold) from **GGGTTA**CTTT**GGGGCA** to **G**CC**TTA**CTTT**G**CC**GCA**. Mice were generated by the transgenic facility at the Oklahoma Medical Research Foundation. Two independent founder lines were obtained and used for experiments.

Mice carrying a conditional knockout of the insulin receptor gene in cardiac tissue (CIRKO) were generated by crossing transgenic mice carrying a floxed insulin receptor allele ($IR^{lox/lox}$) with transgenic mice expressing *cre* recombinase driven by the α-myosin heavy-chain promoter (18). A cross of CIRKO mice and the −895-HG4-CAT mice was carried out to generate a novel transgenic line (CIRKO-HG4-CAT). The new experimental mice have the genotype −895-HG4-CAT-(Cre-$IR^{lox/lox}$), and control littermates have the genotype −895-HG4-CAT-($IR^{lox/lox}$). All mice were kept on a 12-h light/dark cycle in a temperature/humidity-controlled room and had access to free water and standard food. All procedures using animals were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

RNA extraction, quantitative real-time PCR, and RNase protection assay. Mice were killed by CO_2 asphyxiation. Tissues were excised and snap-frozen and RNA was extracted as previously described (16). RNA was quantified by ultraviolet absorbance at 260, 280, and 320 nm.

CAT, mGLUT4, and actin mRNA was quantified by quantitative real-time (qrt)-PCR. The relative copy number of actin was used for normalization. The mRNA levels were calculated using a standard curve of an ultraviolet quantified qrt-PCR sample, specific to the primer set. Primer sequences were as follows: CAT, 5′-TCCGGCAGTTTCTACACATA-3′ (forward), 5′-TGGCTGAGACGAAAAACATA-3′ (reverse); mGLUT4, 5′-AAAAGTGCCTGAAACCAGAG-3′ (forward), 5′-TCACCTCCTGCTCTAAAAGG-3′ (reverse); and actin, 5′-CCTCACTGACTACCTGATGA-3′ (forward), 5′-AGCTCATAGCTCTTCTCCAG-3′ (reverse). All qrt-PCRs were run using an iCycler iQ real-time PCR detection system thermal cycler (Bio-Rad). CIRKO-HG4-CAT sample mRNA was quantified by RNase protection assay as previously described (19).

Metabolic measurements. Blood was collected via tail bleed between 9 and 10 AM from fed animals. Blood glucose was measured using the True-Track smart system glucose meter (Walgreens). Tissue triacylglycerol content was measured using a triglyceride determination kit (T0100; Sigma-Aldrich). Measurement of cAMP levels in adipose tissue was made using a radioimmunoassay kit (Sigma-Aldrich).

Mitochondrial extraction and analysis. Liver and muscle tissues were snap-frozen in liquid nitrogen. Tissues were homogenized in 20 volumes of ice-cold mitochondrial homogenization buffer (10 mmol/l MOPS, pH 7.4, 0.1% BSA). Carnitine palmitoyl transferase activity was assayed in the post-nuclear 500-g supernatant by diluting (1:10 for muscle preparations and 1:40 for liver preparations) in homogenization buffer containing 0.1 mmol/l dithio-nitrobenzoic acid, 0.1 mmol/l palmitoyl CoA, and 10 mmol/l carnitine to a final volume of 1 ml. Change in absorbance at 412 nm wavelength was monitored at 10-s intervals for 1.5 min to estimate the rate of cleavage of palmitoyl CoA.

Luciferase assays. 3T3-L1 adipocytes were differentiated and electroporated as previously described (20). Luciferase assays were performed as previously described with plasmids encoding LXR-α, myocyte enhancer factor (MEF)-2A, and GLUT4 enhancer factor (GEF) 24 h after transfection (21).

Chromatin immunoprecipitation. Three 10-cm plates of 3T3-L1 adipocytes (9 days post-differentiation) were either untreated or treated for 24 h with 25 nmol/l etomoxir, washed in ice-cold 1 × PBS, and fixed at room temperature with 1% formaldehyde in Ham's F-12 starvation media for 10 min. Fixation was stopped by incubation for 5 min with stop-fix solution (125 mmol/l glycine, 11 mmol/l Na_2HPO_4, 1.98 mmol/l KH_2PO_4, 2.97 mmol/l KCl, and 165 mmol/l NaCl). Cells were scraped into 1 × PBS containing 0.5 mmol/l PMSF, pelleted at 1,000 g at 4°C, and then lysed in buffer (5 mmol/l PIPES, pH 8.0, 85 mmol/l KCl, 0.5% NP-40, and protease inhibitors). Crude nuclear pellets were pelleted at 1,000 g at 4°C. The nuclear fraction was resuspended in nuclear lysis buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail). Chromatin was sheared by sonication on ice 10 times (20 s on, 30 s off), and insoluble debris was pelleted at 10,000 g at 4°C. An aliquot representing 10% was analyzed as "input." Supernatant was transferred to a fresh tube and incubated overnight at 4°C with 3 μg of the antibody (anti-LXR [sc-13068X] or normal rabbit IgG, Santa Cruz Biotechnology). Magnetic protein G beads were added the next day and incubated for 4 h, at 4°C. Beads were washed twice with nuclear lysis buffer and four times with wash buffer (100 mmol/l Tris [pH 8.0], 500 mmol/l LiCl, 1% NP-40, 1% deoxycholate). The beads were resuspended in elution buffer (1% SDS, 0.1 mol/l $NaHCO_3$) and incubated at 67°C for 2 h to reverse cross-linking. Beads were pelleted at 12,000 g, and the supernatant was incubated overnight at 67°C to further reverse cross-linking. Supernatant was then cleared by spinning at 10,000*g* and purified using phenol/chloroform extraction and subjected to PCR. GLUT4-specific primers were used to expand the region between domain I and the MEF2 binding domain: GLUT4 forward, 5′-GGAGGGGATGGGCCAGTA; GLUT4 reverse, 5′-GCCCCGAAGTAACCCGGA. The PCR products were separated using 3% agarose gel electrophoresis.

Statistical analysis. Mean and SE of the mean is reported. Differences were analyzed using a two-tailed Student's *t* test.

RESULTS

Insulin resistance and obesity is associated with incomplete fatty acid oxidation (22,23). To explore the consequences of decreased fatty acid oxidation on GLUT4 transcription, we acutely inhibited CPT-1 and thus LCFA entry into the mitochondria. Two-month-old male mice were given one intraperitoneal injection of etomoxir (50 mg/kg body wt). Etomoxir inhibits CPT-1 through an irreversible modification of the active site of CPT-A (24,25). To ensure that etomoxir inhibited CPT-1 activity, we performed a CPT assay in crude mitochondrial preparations from hindquarter skeletal muscle, heart, and liver. CPT-1 activity was inhibited in all tissues tested (Fig. 1*A*).

Fed glucose levels were decreased nearly 50% ($P < 0.05$) after treatment with etomoxir (Fig. 1*B*). Wet weight of perigonadal adipose tissue and subscapular brown adipose tissue were unchanged by etomoxir treatment, suggesting that lipids were not mobilized from these tissues (Fig. 1*C*). Intracellular triacylglycerides were estimated by measuring glycerol content of a total lipid extract after treatment with lipase enzyme. Overnight treatment with etomoxir significantly increased intracellular triacylglycerides in liver tissue and heart ($P < 0.05$) (Fig. 1*D*). By contrast, triacylglycerol content of hindquarter skeletal muscle was significantly decreased in etomoxir-treated mice (Fig. 1*D*).

We then tested *GLUT4* promoter function under etomoxir treatment using a line of transgenic animals that carry the CAT reporter gene expressed under the control of an 895-bp fragment of the human *GLUT4* promoter (−895-HG4-CAT) (Fig. 2*A*). We have previously demonstrated that this fragment of the human *GLUT4* promoter is sufficient to direct tissue-specific and regulated expression of the *CAT* gene in a manner that is identical to endogenous mouse *GLUT4* (16). To this end, CAT and

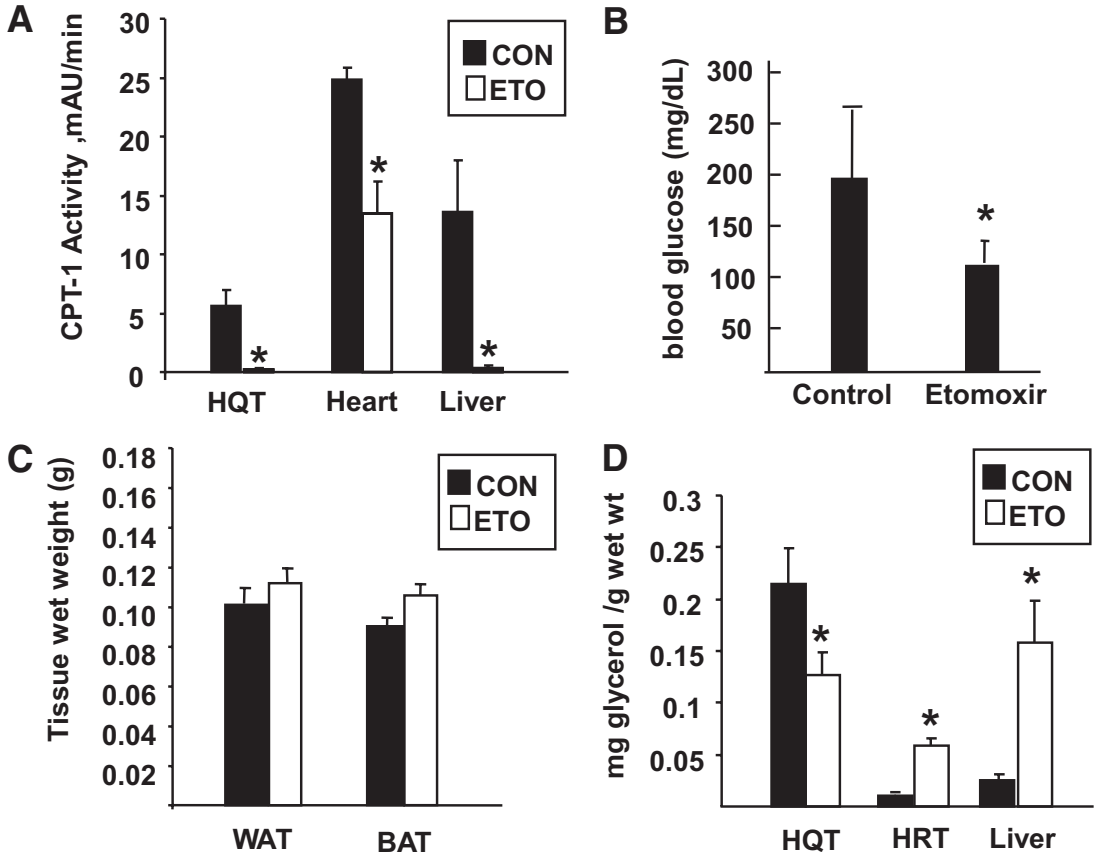

FIG. 1. Effects of etomoxir on CPT-1 activity. The 2- to 3-month-old male mice were given intraperitoneal injections of etomoxir (50 mg/kg body wt). *A*: After 18 h, mitochondria were isolated from hindquarter muscle (HQT), heart, and liver tissues, and CPT-1 activity was measured. *B*: Random fed blood glucose was measured from a tail vein blood sample. *C*: Wet weight of control and etomoxir-treated perigonadal white adipose tissue (WAT) and subscapular brown adipose tissue (BAT) from male mice was determined. *D*: Triacylgyceride was measured in HQT, heart (HRT), and liver after the Folch lipid extraction. All data are presented as mean and SE. Data were analyzed using a two-tailed Student's *t* test. *Significant difference between control (CON) and etomoxir-treated (ETO) ($P < 0.05$).

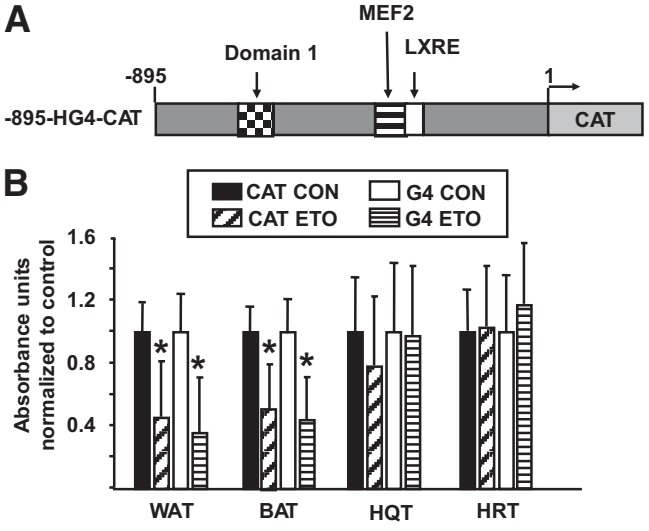

FIG. 2. Effects of etomoxir on *CAT* and *GLUT4* expression. The 2- to 3-month-old male transgenic mice were injected intraperitoneally with 50 mg/kg body wt of etomoxir. *A*: This depicts the schematic drawing of the −895-HG4-CAT construct used to construct this line of transgenic mice. *B*: After 18 h, total RNA was isolated from white adipose tissue (WAT), brown adipose tissue (BAT), hindquarter muscle (HQT), and heart (HRT) tissues. qrt-PCR was used to determine mRNA levels of *CAT*, *GLUT4*, and *actin*. *CAT* and *GLUT4* expression was normalized to *actin* expression levels. Data were analyzed using a two-tailed Student's *t* test. *Significant difference between control (CON) and etomoxir-treated (ETO) ($P < 0.05$).

endogenous GLUT4 mRNA levels were measured in brown adipose tissue and white adipose tissue, hindquarter skeletal muscle, and heart tissue. The effects of etomoxir treatment on *CAT* gene expression and endogenous *GLUT4* gene expression were highly tissue specific. *CAT* mRNA and *GLUT4* mRNA levels in brown adipose tissue and white adipose tissue were decreased ($P < 0.05$) in both male and female mice (Fig. 2*B*). In contrast, *CAT* mRNA and *GLUT4* mRNA in hindquarter skeletal muscle and heart were unaffected by etomoxir treatment (Fig. 2*B*).

Because blood glucose levels were decreased after etomoxir treatment, we reasoned that insulin levels would also be decreased, and this could lead to downregulation of *GLUT4* gene transcription. Using conditional tissue-specific knockouts of the insulin receptor, several studies have shown that *GLUT4* protein is not decreased in adipose tissue, skeletal muscle, or cardiac tissue (18,26,27). To check whether the transcriptional regulation of the *GLUT4* gene was changed by the absence of insulin receptor signaling, we made use of the cardiac-specific receptor knockout of the insulin receptor (CIRKO) model. CIRKO mice were crossed with transgenic mice carrying the −895-HG4-CAT genotype. The CIRKO-HG4-CAT mice were evaluated for their level of *CAT* and endogenous *GLUT4* mRNA expression using the RNAse protection assay. *CAT* and *GLUT4* mRNA expression appear to be unchanged with the loss of an intact insulin receptor (Fig. 3).

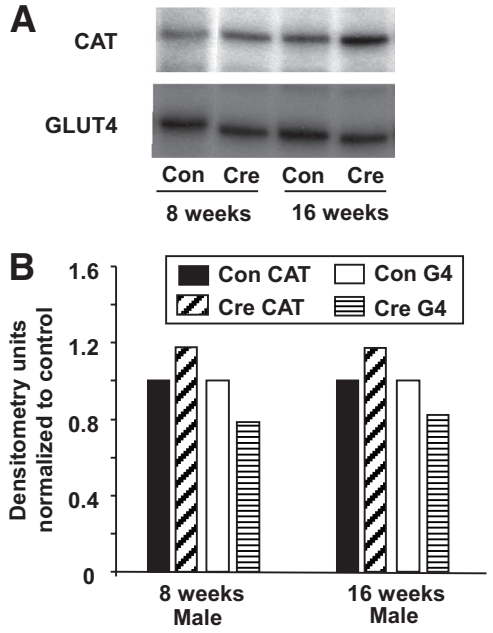

FIG. 3. Effect of the loss of insulin signaling on *CAT* and *GLUT4* transcription in cardiac tissue. *A*: Hearts from CIRKO-hGLUT4-CAT mice (Cre) and wild-type controls (CON) were removed, and total RNA was analyzed for *GLUT4* mRNA and transgenic *CAT* mRNA using RNAse protection assay as described in RESEARCH DESIGN AND METHODS. *B*: Quantification of bands from ^{32}P-labeled probes to the 3′ untranslated region of *CAT* and *GLUT4*. All densitometry measurements were made with ImageJ (National Institutes of Health). The insulin receptor does not directly regulate the levels of *CAT* and *GLUT4* mRNA.

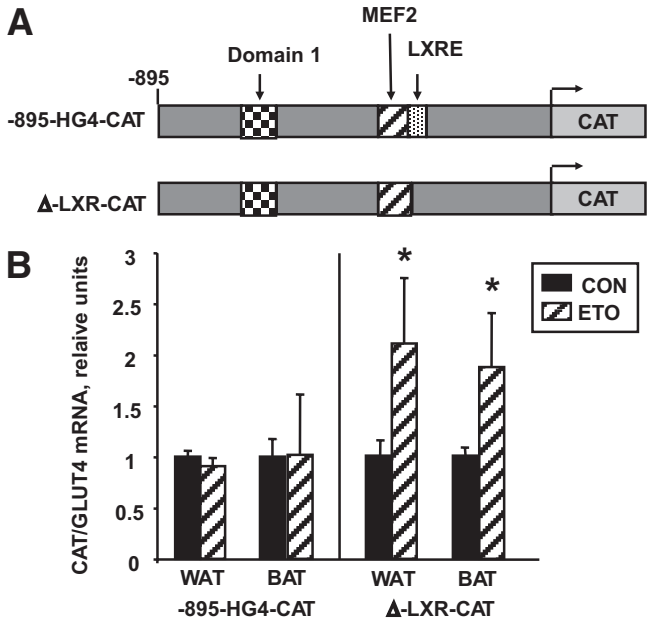

FIG. 4. *GLUT4* LXRE is required for etomoxir-dependent downregulation of *GLUT4* mRNA. *A*: The transgenic constructs used to construct the lines of transgenic mice are shown. *B*: The 2- to 3-month-old transgenic mice were injected intraperitoneally with 50 mg/kg body wt of etomoxir. After 18 h, total RNA was isolated from white adipose tissue (WAT) and brown adipose tissue (BAT) from each control and etomoxir-treated mice. qrt-PCR was used to determine mRNA levels of *CAT*, *GLUT4*, and *actin*. *CAT* and *GLUT4* expression was normalized to actin expression levels. The ratio of *CAT* mRNA to *GLUT4* mRNA was calculated for each sample. Data were analyzed using a two-tailed Student's *t* test. *Significant difference between control (CON) and etomoxir-treated (ETO) ($P < 0.05$).

Because etomoxir treatment inhibits fatty acid import into mitochondria, we reasoned that a signal to the *GLUT4* promoter might be mediated through the putative LXRE on the human *GLUT4* promoter. LXR signaling promotes fat storage; therefore, we reasoned that changes in fat oxidation could be sensed through this signaling pathway (28). To test this hypothesis, we generated a line of transgenic mice carrying a loss of function mutation in the LXRE site in the context of the −895-HG4-CAT transgenic construct. This new mutant construct (Δ-LXR-CAT) was used to generate two founder lines of mice. Both founder lines expressed transgenic mRNA in the GLUT4-expressing tissues, but restricted expression in tissues that do not normally express GLUT4 (data not shown). From this, we conclude that the LXRE site was not required for a tissue-specific expression pattern of the transgene. To determine the role of LXRE in mediating the downregulation of *GLUT4* mRNA under etomoxir treatment, we calculated the ratio of *CAT* mRNA to *GLUT4* mRNA in white and brown adipose treatment in both lines of transgenic mice (Fig. 4). In animals carrying −895-HG4-CAT, the ratio of *CAT* to *GLUT4* remained the same because *CAT* and *GLUT4* mRNA were downregulated to the same extent with etomoxir. In contrast, the ratio of *CAT* mRNA to *GLUT4* mRNA doubled with etomoxir treatment in adipose tissues from animals carrying the Δ-LXR-CAT construct (Fig. 4).

To determine if etomoxir downregulates *GLUT4* through changes in the hormones or other systemic factors other than insulin, we treated 3T3-L1 adipocytes with etomoxir overnight and measured *GLUT4* mRNA (Fig. 5*A*). Similar to adipose tissue in vivo, etomoxir treatment downregulates *GLUT4* mRNA in 3T3-L1 adipocytes about fourfold. To determine if the etomoxir-induced changes were due to inhibition of long-chain fatty acid import, we sought to restore *GLUT4* mRNA by providing palmitoyl-L-carnitine. Etomoxir-treated cells were further treated without or with 1 mmol/l palmitoyl-L-carnitine to bypass carnitine-palmitoyl-transferase activity. This treatment restored *GLUT4* mRNA to 75% of control levels (Fig. 5*B*), confirming that the major effect of etomoxir was due to inhibited formation of acyl carnitines.

In transgenic animals, the signal to downregulate *GLUT4* mRNA was mediated, in part, through the LXRE of the *GLUT4* promoter. To further elucidate LXRE function, we expressed either a luciferase promoter driven by the human *GLUT4* promoter (−895-HG4-LUC) or the construct carrying the loss-of-function mutation in the LXRE (Δ-LXR-HG4-Luc) in 3T3-L1 adipocytes. The constructs were transactivated by overexpression of MEF2A and GEF to boost the signal. Mutation of the LXRE site resulted in a highly reproducible and statistically significant decrease in reporter expression (Fig. 6*A*). When cells were treated with etomoxir, expression of −895-HG4-Luc was decreased ~50% (Fig. 6*A*). Expression of Δ-LXR-HG4-Luc decreased no further upon etomoxir treatment, suggesting that effects of etomoxir treatment are mediated, in part, through LXR signaling. To determine if the LXRE element was bound by LXR protein, we performed chromatin immunoprecipitation using an antibody raised against LXR-α. LXR-α specifically bound the *GLUT4* promoter in control 3T3-L1 adipocytes (Fig. 6*B*); however, LXR-α binding was reduced in etomoxir treatment. Quantification of three independent experiments revealed that etomoxir reduced binding by 84% compared with control cells (see Fig. 7*C* for quantification).

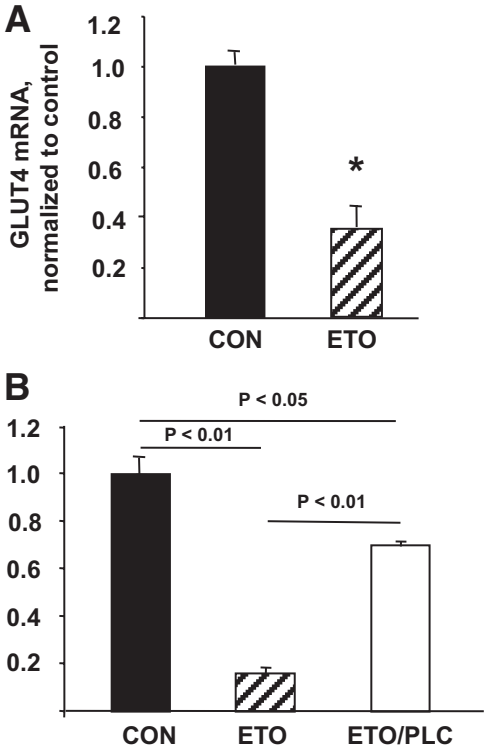

FIG. 5. Effects of etomoxir on *GLUT4* mRNA expression are cell autonomous. 3T3–L1 adipocytes (days 6–8 post-differentiation) were treated with 25 nmol/l etomoxir. *A*: After 18 h, total RNA was isolated and qrt-PCR was used to determine mRNA levels of *GLUT4* and *actin*. *B*: Cells were treated without or with 25 nmol/l etomoxir (ETO) as described above. During the final 4 h of incubation, 1 mmol/l palmitoyl-L-carnitine (PLC) in HEPES-buffered saline, pH 7.4, was added to etomoxir-treated cells. Total RNA was isolated and qrt-PCR was used to determine mRNA levels of *GLUT4* and *actin*. Data were analyzed using a two-tailed Student's *t* test. *Significantly different from control (CON) ($P < 0.05$).

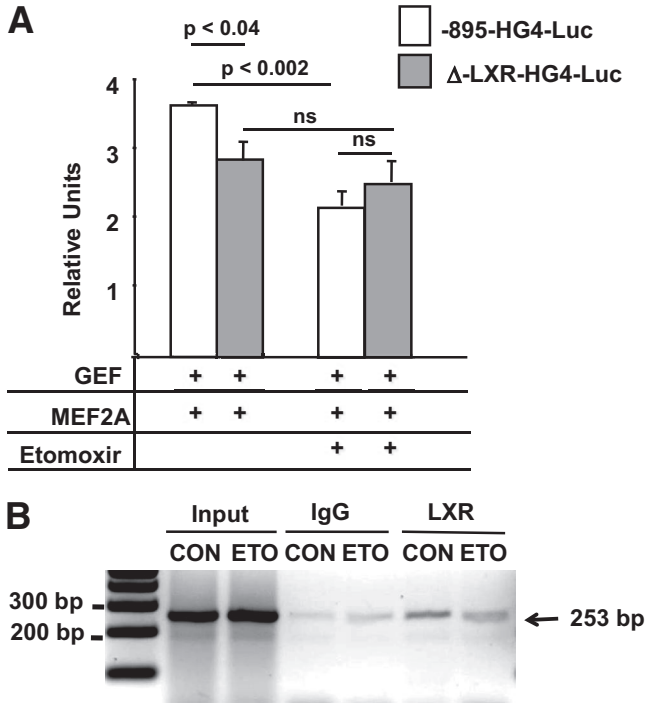

FIG. 6. Role of the *GLUT4* promoter LXRE in GLUT4 gene transcription. *A*: 3T3–L1 adipocytes (day 5 post-differentiation) were transfected with GLUT4 reporter/luciferase (firefly luciferase) reporter plasmids, GLUT4 enhancer factor (GEF) and MEF2 (+ indicates inclusion in transfection), and pRLTKluc (*Renilla* luciferase, for transfection efficiency). Immediately after transfection, the cells were treated without or with 25 nmol/l etomoxir (ETO). After 24 h, cells were harvested and luciferase assays performed in cell lysates. Data were analyzed using a Student's *t* test. Statistical significance between groups is indicated by lines. *B*: Chromatin immunoprecipitation using nuclear extracts from adipocytes (8 days post-differentiation) was performed using an anti–LXR-α antibody and nonimmune IgG. Quantification of this representative chromatin immunoprecipitation is presented in Fig. 7*C*. CON, control.

To understand the nature of the signaling pathway coupled with long-chain acyl carnitine formation, we sought to determine the signaling pathway to activate to restore *GLUT4* mRNA. Because the LXRE element was required for downregulation, we treated cells with the LXR agonist TO901317 (Calbiochem). Treatment with TO901317 partially restored *GLUT4* mRNA to a level that was not statistically lower than control (Fig. 7*A*). To determine if TO901317 treatment influenced LXR binding to the *GLUT4* promoter, we performed chromatin immunoprecipitation using control and etomoxir cell extracts without and with treatment with TO901317. As before, etomoxir treatment reduced LXR-α association with the *GLUT4* promoter (Fig. 7*B* and *C*). Treatment of both control and etomoxir-treated cells with TO901317 significantly enhanced LXR binding to the GLUT4 promoter (Fig. 7*B* and *C*). This suggests that occupancy of the LXRE domain with LXR ligand is required for full activation of *GLUT4* gene expression, but that LXRE ligand occupancy is not rate-setting for *GLUT4* transcription.

DISCUSSION

We have demonstrated that acute inhibition of LCFA import into mitochondria results in a tissue-restricted downregulation of *GLUT4* gene transcription in adipose tissue, but not in skeletal muscle or heart tissue (Fig. 2). Recently, several laboratories have provided evidence supporting a model for dysregulation of lipid metabolism as a cause of insulin resistance (23,29,30). These papers invoke several mechanisms including increased levels of ceramide (30), increased levels of the signaling and biosynthetic intermediate diacylglycerol (29), and reduced capacity for mitochondrial oxidation of fatty acids (23) as the cause of insulin resistance. Because the level of *GLUT4* expression may play a role in chronic insulin resistance, it seems likely that changes in lipid metabolism could regulate *GLUT4* transcription and contribute to downregulation of *GLUT4* mRNA. We reasoned that inhibition of mitochondrial LCFA might also play a role in generating a metabolic signal leading to regulation of *GLUT4* transcription. We observed that the acute inhibition of mitochondrial LCFA inhibited *GLUT4* transcription in white and brown adipose tissue, but did not alter transcription in skeletal muscle or heart (Fig. 2). This suggests that inhibition of LCFA import, per se, does not directly inhibit *GLUT4* transcription, but rather the fate of the imported LCFA in a given tissue may generate a signal to the *GLUT4* promoter. Therefore, the effect of etomoxir on adipose tissue reveals a unique function for mitochondrial LCFA import in this tissue.

In our model, intracellular triacylglycerol accumulated in liver and heart tissue, but not skeletal muscle (Fig. 1). This was unexpected, since previous studies showed that etomoxir treatment in rats led to increased triacylglycerol in skeletal muscle (31). The different findings in the C57BL/6 mice used in this study compared with rats

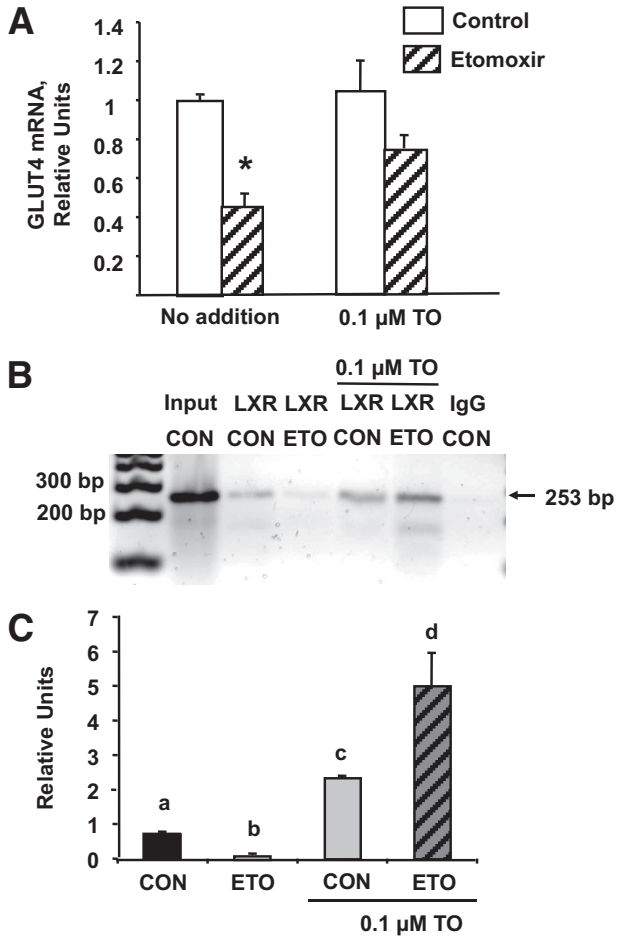

FIG. 7. Treatment with LXR agonist recovers GLUT4 mRNA expression in etomoxir-treated cells. *A*: 3T3–L1 adipocytes (day 6–8 post-differentiation) were treated with 25 nmol/l etomoxir without or with 0.1 μmol/l TO901317. After 18 h, total RNA was isolated for quantification of GLUT4 and actin mRNA. *B*: Chromatin immunoprecipitation (ChIP) using nuclear extracts from adipocytes (8 days post-differentiation) was performed using an anti–LXR-α antibody and nonimmune IgG. Cells were treated without and with 25 nmol/l etomoxir (ETO) and/or 0.1 μmol/l TO901317 as described in *A*. *C*: Quantification of three independent ChIP experiments using qrt-PCR. IgG background is subtracted from the LXR antibody ChIPs. Data were analyzed using two-tailed Student's *t* test. Bars with different lowercase letters are significantly different from one another ($P < 0.05$). CON, control.

suggest a species or strain-specific difference in fatty acid metabolism. For instance, the C57BL/6 mice may have increased secretion of free fatty acids and triacylglycerol from skeletal muscle or decreased uptake of free fatty acids from adipose tissue and liver. Nonetheless, the accumulation of triacylglycerol in heart tissue did not correlate with a downregulation of *GLUT4* transcription, suggesting that these events are not directly linked. This is somewhat surprising to us, since increased triacylglycerol levels in hearts of mice with a cardiac-specific overexpression of lipoprotein lipase correlated with decreased *GLUT4* mRNA levels (32). That study might reflect a chronic adaptation to continued high levels of triacylglycerol in those transgenic mice.

In humans and rodents, diabetes and obesity lead to downregulation of *GLUT4* gene transcription in adipose tissue (19,33–35). The signaling pathways that are responsible remain elusive. GLUT4 expression in adipose tissue is also downregulated in response to prolonged fasting. Downregulation of adipose GLUT4 in diabetes and fasting are consistent with the notion that *GLUT4* transcription is regulated by decreased insulin signaling, increased counterregulatory hormone secretion, or the concomitant changes in substrate flux that accompany both insulin resistance and fasting (34,36). For example, during insulin resistance and fasting, insulin signaling is reduced, insulin-mediated glucose flux is low, and circulating fatty acids are high. Insulin resistance and fasting have similar effects on adipose tissue metabolism, including increased lipolysis with release of fatty acids and glycerol that is used for hepatic gluconeogenesis (37). On the other hand, insulin resistance states and fasting have different effects on substrate utilization in skeletal muscle. Fatty acid oxidation is inhibited in insulin resistance, while it is quite active in fasting (22,23,38). In muscles of obese and type 2 diabetics, glucose oxidation in the fasted state in much higher than lean controls, and there is little or no difference in fatty acid oxidation between the fed and fasted states (38).

We have now used a metabolic paradigm that incorporates aspects of both enhanced glucose utilization of plasma glucose levels and decreased lipid utilization to examine the effects on *GLUT4* expression. Etomoxir-treatment inhibited β-oxidation by inhibiting CPT-1 activity and lowered blood glucose levels (Fig. 1). Previous studies in rats have shown that etomoxir treatment lowers insulin levels, but does not necessarily lead to lipolysis, since the fat mass was reported to be increased and circulating free fatty acids not elevated (31). In the current study, we found that neither adipose tissue wet weight (Fig. 1*C*) nor cAMP levels (data not shown) were altered under etomoxir treatment. Nevertheless, the *GLUT4* transcription levels in both brown and white adipose tissue were reduced under etomoxir treatment consistent with a shift away from glucose utilization in adipose tissue, thereby increasing its availability for other organs.

Although we have not shown it directly, it is unlikely that the downregulation of *GLUT4* transcription in adipose tissue is due to low levels of signaling through the insulin receptor. First, the adipose-specific knockout of the insulin receptor does not alter GLUT4 protein expression (18). Second, we demonstrated that GLUT4 transcription in heart is not altered by cardiac-specific knockout of the insulin receptor (Fig. 3). While we did not find that etomoxir led to changes in cardiac *GLUT4* gene expression, the findings that insulin receptor signaling is not required for *GLUT4* transcription in the cardiac tissue is evidence that other signals likely regulate the *GLUT4* promoter.

GLUT4 transcription is differentially regulated in adipose tissue compared with muscle tissue. The differences in transcription likely reflect differences in the physiologic and metabolic roles that each of these tissues play. In adipose tissue, GLUT4 may be playing a role in energy sensing, whereas in muscle tissue, GLUT4 may play a more important role in fuel selection. This is supported by evidence that *GLUT4* knockout mice oxidize fatty acids at a significantly higher rate than wild-type mice (39,40). Moreover, skeletal muscle and adipose tissue respond differently to activation of AMP-activated protein kinase (AMPK). In skeletal muscle, AMPK activation leads to increased glucose uptake and fatty acid oxidation presumably to increase ATP levels, while in adipose tissue, AMPK activation inhibits these processes, presumably allowing free fatty acids to be released from adipocytes for use in other tissues (41,42). In part, the effects of AMPK on

skeletal muscle metabolism may be mediated through increased *GLUT4* gene transcription in muscle (43,44).

GLUT4 transcription is regulated by metabolic changes that are associated with insulin resistance, but might not be directly regulated by insulin action per se. We demonstrated that the effects of etomoxir (in vivo and in vitro) are mediated, in part, by LXR signaling (Figs. 4, 6, and 7). The GLUT4 promoter regulatory region contains an LXRE site, which we have now confirmed binds to the LXR-α ligand both in adipose tissue, and 3T3-L1 adipocytes (Figs. 6 and 7). Similar to insulin, LXR signaling promotes the synthesis and storage of lipids (28). It is quite reasonable that LXR signaling would support production of *GLUT4* mRNA during times when lipid storage in adipose tissue is required. Etomoxir treatment reduced LXR-α association with the *GLUT4* promoter, consistent with the conclusion that LXR plays a positive role in *GLUT4* gene transcription through the LXRE.

Replenishment of etomoxir-treated cells with either palmitoyl L-carnitine, the product of CPT-1 enzyme, and LXR agonist each restored *GLUT4* expression to near-normal levels, suggesting that mitochondrial import of long-chain fatty acids may play in regulating LXR signaling in the adipocyte. This effect of LXR activation was present in cultured cells, suggesting that systemic factors are not involved. Thus, a metabolite of palmitate oxidation may generate the LXR ligand. Conversely, fatty acid oxidation may indirectly produce a LXR ligand. In hepatic cells, LXR may function as a glucose sensor by directly binding glucose or glucose-6-phosphate to induced LXR-dependent transcriptional activation (45). Finally, inhibition of fatty acid oxidation may lead to increased AMPK activation in adipose tissue, as is observed in fasted adipocytes (46), which in turn leads to decreased glucose uptake, lipogenesis, and fatty acid oxidation (41). In hepatic tissue, AMPK activation has been shown to suppress LXR-dependent gene transcription presumably through alterations in ligand production. It is possible that this mechanism also operates in adipose tissue (47). In fact, changes in LXR signaling may be part of the transition between the fed and fasted state in adipose tissue and show that LXR is responding to glucose flux in adipose tissue. LXR signaling may play a role in the adaptation to the metabolic stress that accompanies over-nutrition and diabetes. For example, treatment of obese insulin-resistant C57BL/6 mice with an LXR agonist improved glucose homeostasis (48). Future work is underway to determine the role of LXR signaling to the *GLUT4* promoter in states of relative insulin deficiency.

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No potential conflicts of interest relevant to this article were reported.

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