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Constitutively Active CaMKKα Stimulates Skeletal Muscle Glucose Uptake in Insulin-Resistant Mice In Vivo

In insulin-sensitive skeletal muscle, the expression of constitutively active Ca^{2+}/calmodulin-dependent protein kinase kinase α (caCaMKKα) stimulates glucose uptake independent of insulin signaling (i.e., Akt and Akt-dependent TBC1D1/TBC1D4 phosphorylation). Our objectives were to determine whether caCaMKKα could stimulate glucose uptake additively with insulin in insulin-sensitive muscle, in the basal state in insulin-resistant muscle, and if so, to determine whether the effects were associated with altered TBC1D1/TBC1D4 phosphorylation. Mice were fed a control or high-fat diet (60% kcal) for 12 weeks to induce insulin resistance. Muscles were transfected with empty vector or caCaMKKα plasmids using in vivo electroporation. After 2 weeks, caCaMKKα protein was robustly expressed. In insulin-sensitive muscle, caCaMKKα increased basal in vivo [^{3}H]-2-deoxyglucose uptake approximately twofold, insulin increased glucose uptake approximately twofold, and caCaMKKα plus insulin increased glucose uptake approximately fourfold. caCaMKKα did not increase basal TBC1D1 (Ser^{237}, Thr^{590}, Ser^{660}, pan-Thr/Ser) or TBC1D4 (Ser^{588}, Thr^{642}, pan-Thr/Ser) phosphorylation. In insulin-resistant muscle, caCaMKKα increased basal glucose uptake approximately twofold, and attenuated high-fat diet–induced basal TBC1D1 (Thr^{590}, pan-Thr/Ser) and TBC1D4 (Ser^{588}, Thr^{642}, pan-Thr/Ser) phosphorylation. In cell-free assays, CaMKKα increased TBC1D1 (Thr^{590}, pan-Thr/Ser) and TBC1D4 (Ser^{588}, pan-Thr/Ser) phosphorylation. Collectively, these results demonstrate that caCaMKKα stimulates glucose uptake additively with insulin, and in insulin-resistant muscle, and alters the phosphorylation of TBC1D1/TBC1D4.

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Skeletal muscle is the primary site for insulin-stimulated glucose disposal in the human body, accounting for 80–90% of all the glucose taken up from the blood (1). In people with type 2 diabetes, while the ability of insulin to stimulate muscle glucose uptake is impaired (2), the ability of non-insulin–dependent stimuli, such as

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exercise/muscle contraction, to increase glucose uptake remains intact (3). Thus, determination of the intracellular signaling mechanisms underlying insulin-independent muscle glucose uptake may identify novel pharmaceutical targets for the treatment of type 2 diabetes.

Intracellular Ca^{2+} plays a critical role in numerous cellular and metabolic processes in muscle, including the regulation of contractile activity and glucose uptake. Importantly, studies have shown that stimulation of isolated rodent muscles with low doses of the sarcoplasmic reticulum Ca^{2+} release agents, caffeine or *N*-(6-amino-hexyl)-5-chloro-1-napthalenesulfonamide (W-7), can increase glucose uptake twofold to fourfold, independent of detectable force development (4–6), changes in cellular energetics (4), or energetic downstream signals (i.e., AMP-activated protein kinase [AMPK]) (5), demonstrating that Ca^{2+}-dependent signaling is an independent pathway regulating muscle glucose uptake.

The signal transduction pathways by which changes in intracellular Ca^{2+} levels stimulate muscle glucose uptake are not well understood, although studies have suggested a role for the Ca^{2+}-activated, serine/threonine kinase, Ca^{2+}/calmodulin-dependent protein kinase kinase α (CaMKKα) in this process. Previous work in rodent muscle has shown that inhibition of CaMKK signaling with the chemical CaMKK inhibitor STO-609 significantly inhibited contraction-induced glucose uptake, independent of detriments in force production (7,8). In addition, the expression of a constitutively active form of CaMKKα in insulin-sensitive muscle stimulated glucose uptake by ~2.5-fold and occurred independently of the phosphorylation of key insulin-signaling proteins (i.e., the serine/threonine kinase, Akt [Thr^{308}] and the Rab GTPase activating proteins TBC1D1/TBC1D4 [phospho-Akt substrate (PAS)]) (7). Collectively, these studies suggest a key potential role for CaMKK in the regulation of insulin-independent muscle glucose uptake. Despite this evidence, to date, no studies have examined whether activation of CaMKKα signaling plus insulin stimulation have additive effects on muscle glucose uptake, or whether activation of CaMKKα signaling can stimulate glucose uptake in a model of muscle insulin resistance. Thus, the objectives of this study were to determine whether constitutive activation of CaMKKα signaling could stimulate glucose uptake additively with insulin, or in insulin-resistant muscle, and if so whether this occurs via the phosphorylation of Akt, TBC1D1, and/or TBC1D4.

RESEARCH DESIGN AND METHODS

Animals

Experiments were performed in accordance with the East Carolina University Institutional Animal Care and Use Committee and the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. Mice were housed in cages at 21–22°C with a 12 h light/dark cycle. Male C57BL/6J mice were fed a chow diet or a 60 kcal% high-fat diet (Research Diets) starting at 6 weeks of age, and obtained from The Jackson Laboratory (Bar Harbor, ME) at 16–17 weeks of age. Mice were maintained on a chow diet containing 14 kcal% fat (Prolab RMH 3000; PMI Nutritional International) or the 60 kcal% fat diet for the remainder of the study. Food and water were available ad libitum.

Assessment of Insulin Resistance

Mice on the diets for 12 weeks were fasted overnight (~14 h), and blood was taken to assess glucose levels using a glucometer (LifeScan, Inc.); insulin levels were determined by ELISA (EMD Millipore). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by multiplying fasted blood glucose (in millimoles per liter) by fasted blood insulin levels (in milliunits per liter) and dividing by 22.5. Control and high-fat–diet mice were randomly divided into three groups to assess ex vivo muscle glucose uptake, in vivo muscle glucose uptake, or muscle intracellular signaling proteins. Body weight, blood glucose, blood insulin, and HOMA-IR levels are provided for each group separately in Supplementary Table 1.

Ex Vivo Skeletal Muscle [^{3}H]-2-Deoxyglucose Uptake

Ex vivo muscle glucose uptake was performed using methods adapted from Witczak et al. (9). Briefly, mice were fasted overnight, anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneal), and killed by cervical dislocation. The extensor digitorum longus muscles were removed and placed in gassed 37°C Krebs-Ringer bicarbonate (KRB) solution containing the following (in mmol/L): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 KH_2PO_4, 1.2 $MgSO_4 \cdot 7H_2O$, and 24.6 $NaHCO_3$ supplemented with 2 pyruvate for 60 min. Muscles were incubated in KRB plus pyruvate containing 0 or 4,167 pmol/L (600 μU/mL) insulin (Roche Diagnostics) for 20 min, then in KRB buffer containing 1.5 μCi/mL [^{3}H]-2-deoxyglucose, 1 mmol/L deoxyglucose, 0.45 μCi/mL [^{14}C]-mannitol, and 7 mmol/L mannitol with or without insulin. Muscles were frozen in liquid N_2, solubilized in 1N NaOH at 80°C and neutralized with 1N HCl. Samples were centrifuged at 11,000*g* for 1 min. Aliquots were removed for scintillation counting of the [^{3}H] and [^{14}C] labels, and [^{3}H]-deoxyglucose uptake calculated.

Transfection of Mouse Muscle Using In Vivo Electroporation

Plasmids containing truncated constitutively active CaMKKα (caCaMKKα) (amino acids 1–434) or empty vector pCS2+ were donated by Thomas R. Soderling (Vollum Institute, Oregon Health and Science University, Portland, OR) (10). Plasmid DNA injections and in vivo electroporation were performed as previously described (7,9). For all transfections, plasmid DNA for active CaMKKα was injected into the tibialis anterior muscle of one leg, and empty vector was injected into the contralateral muscle. Muscles were allowed 2 weeks to express proteins.

In Vivo Skeletal Muscle [^{3}H]-2-Deoxyglucose Uptake

In vivo muscle glucose uptake was assessed using methods adapted from Witczak and colleagues (7,9). Mice were fasted overnight and anesthetized with pentobarbital sodium. After 30 min, blood was taken from the tail to assess glucose, insulin, and background radioactivity. A bolus of [^{3}H]-2-deoxyglucose (0.33 μCi [^{3}H]/g body weight) dissolved in 0.9% NaCl (4 μL/g body weight) or 20% glucose (1 mg glucose/g body weight) was administered retro-orbitally, and blood was taken 5, 10, 15, 25, 35, and 45 min later for glucose, [^{3}H]-2-deoxyglucose, and/or insulin measurements. Mice were killed and muscles frozen in liquid N_2. Muscles were weighed and homogenized in buffer containing the following (in mmol/L): 20 Tris-HCl, pH 7.5, 5 EDTA, 10 $Na_4P_2O_7$, 100 NaF, 2 $NaVO_4$, 0.01 leupeptin, 3 benzamidine, 1 phenylmethylsulfonyl fluoride, and 10 μg/mL aprotinin. Homogenates were divided for [^{3}H]-2-deoxyglucose uptake and immunoblot analyses. Accumulation of muscle radioactivity was assessed using procedures modified from Ferré et al. (11), and the rate of glucose uptake was calculated as previously described (12). Homogenates not used for uptake measurements were mixed with 1% Nonidet P-40 and were processed for immunoblots as described below.

Assessment of Intracellular Signaling by Immunoprecipitation and Immunoblot Analysis

Intracellular signaling was assessed in muscle expressing caCaMKKα, as previously described (7). Mice were fasted overnight and anesthetized with pentobarbital sodium. After 30 min, blood was taken from the tail to assess glucose and insulin levels. A bolus of 0.9% NaCl was administered retro-orbitally, and blood was taken 10 min later for glucose and insulin measurements. Mice were killed, and muscles were frozen in liquid N_2. Muscles were homogenized in buffer containing the following (in mmol/L): 20 Tris-HCl, pH 7.5, 5 EDTA, 10 $Na_4P_2O_7$, 100 NaF, 2 $NaVO_4$, 1% Nonidet P-40, 0.01 leupeptin, 3 benzamidine, 1 phenylmethylsulfonyl fluoride, and 10 μg/mL aprotinin. Samples were rotated end over end at 4°C for 1 h and centrifuged at 14,000*g* for 30 min. Protein concentrations were determined via the Bradford method.

Immunoprecipitation was performed using standard methods (7). Briefly, muscle lysates (400 μg for TBC1D1; 800 μg for TBC1D4) were incubated with TBC1D1 or TBC1D4 antibodies and protein G beads in buffer containing the following (in mmol/L): 20 Tris-HCl, pH 7.6, 200 NaCl, 5 EDTA, 1% Triton X-100, 10 μg/mL aprotinin, 0.01 leupeptin, 3 benzamidine, 1 phenylmethylsulfonyl fluoride, 100 NaF, and 2 $NaVO_4$ at 4°C. The supernatant was removed, and beads were washed four times. Laemmli buffer was added, and samples were heated at 95°C for 5 min.

Immunoblots were performed using standard methods (13). Briefly, muscle lysates (20–60 μg) or immunoprecipitates were subjected to SDS-PAGE, and proteins were transferred onto nitrocellulose membranes and then incubated with one of the following primary antibodies: phospho-Akt (Thr^{308}), phospho-Akt (Ser^{473}), pan-Akt, phospho-AMPK (Thr^{172}), AMPKα, phospho-TBC1D1 (Thr^{590}), phospho-TBC1D1 (Ser^{660}), TBC1D1, phospho-TBC1D4 (Ser^{588}), phospho-TBC1D4 (Thr^{642}), phospho-pan-Thr (Thr/Ser) from Cell Signaling Technology; GLUT1, GLUT4, phospho-TBC1D1 (Ser^{237}), TBC1D4 from Millipore; and CaMKKα (F-2), Hexokinase II from Santa Cruz Biotechnology. Horseradish peroxide–conjugated secondary antibodies were incubated with the membrane and detected using chemiluminescence reagents (PerkinElmer). Densitometric analysis was performed using Image Lab software (Bio-Rad).

Cell-Free Phosphorylation Assays

Myc-tagged human TBC1D1 or TBC1D4 (OriGene Technologies) proteins (0.3 μg) were incubated in buffer containing the following (in mmol/L): 50 HEPES, pH 7.4, 1 EGTA, pH 8.0, 1 dithiothreitol, 10 Mg-acetate · $4H_2O$, and 0.1 ATP, with or without 7 μmol/L calmodulin and 4 mmol/L $CaCl_2$, with or without 0.1 μg human CaMKKα isoform a (SignalChem) at 37°C for 30 min. Reactions were terminated by the addition of Laemmli buffer and heated at 95°C for 5 min. Reaction mixtures were subjected to immunoblot analysis as described above.

Statistical Analysis

Data are presented as the mean ± SEM. Statistical significance was defined as $P < 0.05$ and was determined by Student *t* tests or two-way ANOVA and Student-Newman-Keuls post hoc analysis. The number of mice or muscles used to determine significance is indicated in the text or figure legends.

RESULTS

caCaMKKα Plus Insulin Has Additive Effects on Muscle Glucose Uptake

Previous work has shown that the expression of caCaMKKα in insulin-sensitive muscle increased glucose uptake ~2.5-fold independent of insulin signaling (7), suggesting that CaMKKα may play a critical role in the regulation of insulin-independent muscle glucose uptake. However, to date, no studies have examined whether expression of caCaMKKα and insulin have additive effects on muscle glucose uptake. To address this goal, muscles from insulin-sensitive mice were transfected with plasmids containing empty vector or caCaMKKα using in vivo electroporation. Two weeks later, in vivo muscle glucose uptake was assessed in the basal state or in response to a physiological insulin response (i.e., intravenous glucose injection). As shown in Fig. 1*A* and *B*, intravenous glucose

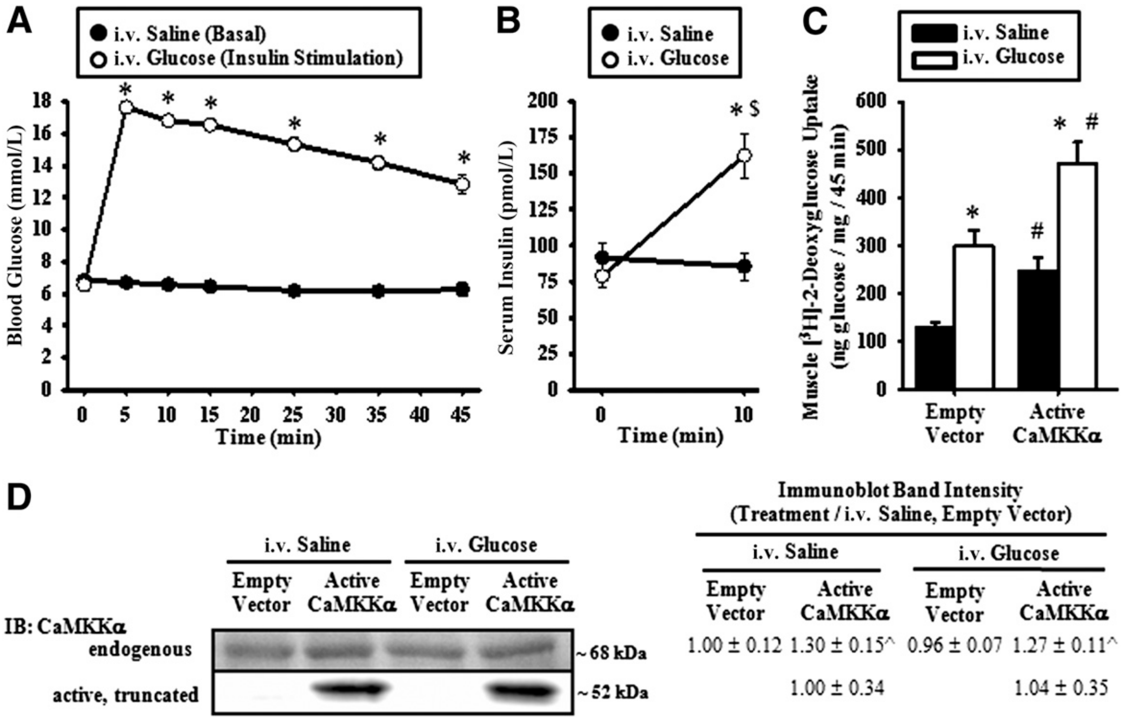


Figure 1—Expression of caCaMKKα and insulin stimulation additively increase glucose uptake in mouse skeletal muscle in vivo. Blood glucose (*A*) and serum insulin (*B*) levels were assessed for 45 min following a retro-orbital injection of [^{3}H]-2-deoxyglucose dissolved in saline (intravenous saline) or a 20% glucose solution (intravenous glucose) to elicit insulin release from the pancreas. *C*: Muscle glucose uptake was assessed in tibialis anterior muscles transfected with empty vector or caCaMKKα for 2 weeks. *D*: Representative immunoblots and quantification to assess caCaMKKα and endogenous CaMKKα expression. Statistical significance was defined as $P < 0.05$: *vs. intravenous saline (basal); $vs. 0 min; #vs. empty vector; ^main treatment effect vs. empty vector. n = 12–13 mice or muscles per treatment group. IB, immunoblot.

significantly increased blood glucose and insulin levels, demonstrating that these procedures elicit insulin secretion from the pancreas. caCaMKKα increased basal glucose uptake approximately twofold (Fig. 1*C*), while insulin-stimulated glucose uptake increased approximately twofold (Fig. 1*C*), consistent with previous findings in insulin-sensitive muscle (7,9). The combination of caCaMKKα plus insulin increased glucose uptake approximately fourfold (Fig. 1*C*), demonstrating that activation of CaMKKα signaling plus insulin can additively increase glucose uptake into muscle. To assess whether CaMKKα expression was the same in both treatment groups, immunoblots were performed. Importantly, in the same muscles used to assess glucose uptake, there was no significant difference in caCaMKKα expression (Fig. 1*D*). The ratio of caCaMKKα to endogenous CaMKKα was assessed in immunoblots at the exact same exposure time, and was calculated to be 55.2 ± 7.3 and 69.8 ± 10.6 in the muscles from the saline-injected and glucose-injected mice, respectively. It was not statistically different (P = 0.276) between the treatment groups.

High-Fat Diet–Induced Skeletal Muscle Insulin Resistance

Since caCaMKKα plus insulin had additive effects on muscle glucose uptake, next it was important to determine whether caCaMKKα could stimulate glucose uptake in insulin-resistant muscle. To generate a model of muscle insulin resistance, male C57BL/6J mice were fed a high-fat diet for 12 weeks. As shown in Table 1, mice receiving a high-fat diet exhibited significant

Table 1—12-Week high-fat diet induces insulin resistance in male C57BL/6J mice

Characteristics	Treatment group: Control diet (*N* = 37)	Treatment group: High-fat diet (*N* =38)
Age (weeks)	18 ± 0	18 ± 0
Body weight (g)	26.2 ± 0.3	34.8 ± 0.7*
Fasted blood glucose (mmol/L)	6.7 ± 0.3	9.6 ± 0.4*
Fasted serum insulin (pmol/L)	53.8 ± 3.3	121.9 ± 15.2*
HOMA-IR (mmol/L × mU/L)	2.2 ± 0.1	7.3 ± 1.0*

Mice were fed either a low-fat (control) or a high-fat (60 kcal%) diet for 12 weeks starting at 6 weeks of age. After an overnight fast, mice were weighed and blood samples were taken to assess glucose and insulin levels, and to calculate HOMA-IR. *Statistical significance was defined as $P < 0.05$ vs. control diet.

increases in blood glucose and insulin levels, and HOMA-IR, demonstrating that the high-fat diet–induced systemic insulin resistance. To examine muscle insulin resistance, insulin-stimulated ex vivo muscle glucose uptake was measured in a subset of mice. As shown in Fig. 2, muscles from the high-fat–fed mice exhibited significantly impaired insulin-stimulated glucose uptake confirming muscle insulin resistance in these animals.

caCaMKKα Stimulates Glucose Uptake in Insulin-Resistant Muscle

To determine whether activation of CaMKKα signaling could stimulate glucose uptake in insulin-resistant muscle, muscles from control and high-fat diet–fed mice were transfected with empty vector or caCaMKKα. Two weeks later, mice were injected with [^{3}H]-2-deoxyglucose to assess muscle glucose uptake. As shown in Fig. 3*A* and *B*, the intravenous saline injection did not elicit a significant change in blood glucose or insulin levels in either the control or high-fat diet–fed mice. In both the control and high-fat–fed mice, caCaMKKα increased muscle glucose uptake by approximately twofold (Fig. 3*C*), demonstrating that the activation of CaMKKα signaling can increase glucose uptake in both insulin-sensitive and insulin-resistant muscle. Immunoblots were performed to assess CaMKKα protein levels, and there was no significant difference in caCaMKKα expression (Fig. 3*D*). The mean ratios of caCaMKKα to endogenous CaMKKα were 43.0 ± 14.2 and 74.7 ± 18.5 in the muscles from the control and high-fat diet–fed mice, respectively, and were not statistically different (P = 0.207).

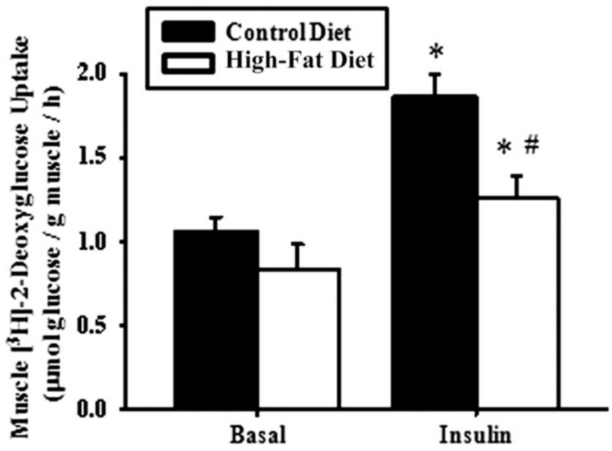


Figure 2—A 12-week high-fat diet induces skeletal muscle insulin resistance in male C57BL/6J mice. In mice fed either a control diet or a high-fat diet for 12 weeks, muscle glucose uptake was assessed in isolated extensor digitorum longus muscles in response to insulin (4,167 pmol/L = 600 μU/mL). Statistical significance was defined as $P < 0.05$: *vs. basal; #vs. control diet. n = 6–8 muscles per treatment group.

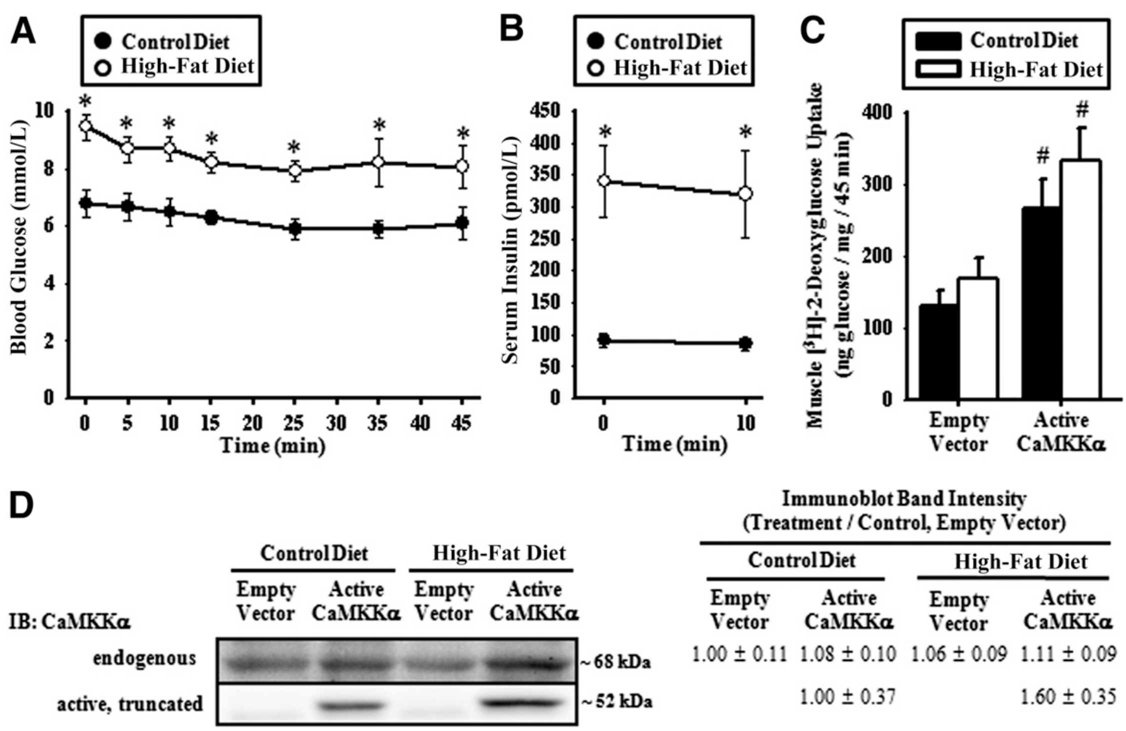


Figure 3—Expression of caCaMKKα stimulates glucose uptake in the skeletal muscle of insulin-resistant mice in vivo. Blood glucose (*A*) and serum insulin (*B*) levels were assessed for 45 min after a retro-orbital injection of [^{3}H]-2-deoxyglucose dissolved in saline. *C*: Muscle glucose uptake was assessed in tibialis anterior muscles transfected with empty vector or caCaMKKα for 2 weeks. *D*: Representative immunoblots and quantification to assess caCaMKKα and endogenous CaMKKα expression. Statistical significance was defined as $P < 0.05$: *vs. control diet; #vs. empty vector. n = 7–8 mice or muscles per treatment group. IB, immunoblot.

caCaMKKα Does Not Stimulate TBC1D1/TBC1D4 Phosphorylation on Akt- or AMPK-Dependent Sites in Muscle

Insulin stimulates muscle glucose uptake via a relatively well-characterized signaling cascade, involving the phosphorylation of Akt, TBC1D1, and TBC1D4 (reviewed in Sakamoto and Holman [14]). Previous work in insulin-sensitive muscle showed that caCaMKKα stimulated glucose uptake independent of an increase in Akt (Thr^{308}) and TBC1D1/TBC1D4 (PAS) phosphorylation (7), suggesting that CaMKKα stimulates glucose uptake independent of insulin signaling. However, since the publication of that manuscript a large number of phosphorylation sites have been identified on TBC1D1 and TBC1D4 that have been implicated in the regulation of muscle glucose uptake, and specific antibodies made against some of them. Thus, it was next important to determine whether caCaMKKα increased glucose uptake in insulin-resistant muscle via phosphorylation of Akt, TBC1D1, or TBC1D4.

As shown in Fig. 4, in the muscles used for intracellular signaling, the ratio of active to endogenous CaMKKα was 32.7 ± 3.8 (control diet) and 29.5 ± 2.6 (high-fat diet), and was not statistically different (P = 0.496). In both the control and insulin-resistant muscles, caCaMKKα did not increase the basal phosphorylation of Akt (Thr^{308}) or (Ser^{473}), pan-Akt expression, or the ratio of phosphorylated to total pan-Akt (Fig. 4). Thus, phosphorylation of Akt is not the mechanism for CaMKKα-induced glucose uptake. To examine whether CaMKKα could be stimulating glucose uptake via TBC1D1 or TBC1D4 phosphorylation on known Akt sites, immunoblots were performed to assess TBC1D1 (Thr^{590}), TBC1D4 (Ser^{588}), and TBC1D4 (Thr^{642}) phosphorylation, as well as TBC1D1 and TBC1D4 protein levels. In both the control and insulin-resistant muscles, caCaMKKα did not increase TBC1D1 or TBC1D4 protein expression or basal phosphorylation on any of these sites (Fig. 4). In the insulin-resistant muscles, there was a significant increase in the basal phosphorylation of TBC1D1 (Thr^{590}) and TBC1D4 (Ser^{588}) that was attenuated by caCaMKKα (Fig. 4), suggesting that CaMKKα-mediated signaling could be inducing TBC1D1/TBC1D4 phosphorylation on sites that compete with other phosphorylation sites.

AMPK is a known substrate of CaMKKα, and previous work has shown that caCaMKKα expression stimulated an approximately twofold increase in AMPK (Thr^{172}) phosphorylation and activity in insulin-sensitive muscle (7). Since AMPK can phosphorylate TBC1D1 (Ser^{237}, Ser^{660}) and TBC1D4 (Ser^{711}), we next examined whether active CaMKKα could be inducing TBC1D1/TBC1D4 phosphorylation on AMPK-dependent sites. Since there are currently no commercially available antibodies for phosphorylated TBC1D4 (Ser^{711}), only phosphorylated TBC1D1 (Ser^{237}, Ser^{660}) was examined. In control muscles under basal conditions (i.e., without glucose stimulation), despite an approximately twofold increase in AMPK (Thr^{172}) phosphorylation, caCaMKKα did not increase TBC1D1 (Ser^{237}) or (Ser^{660}) phosphorylation (Fig. 4). In the insulin-resistant muscles under basal conditions, there was a significant increase in the basal phosphorylation of TBC1D1 (Ser^{237}) and TBC1D1 (Ser^{660}), and this increase was not affected by caCaMKKα (Fig. 4). Thus, caCaMKKα is not stimulating TBC1D1/TBC1D4 phosphorylation on AMPK-dependent sites.

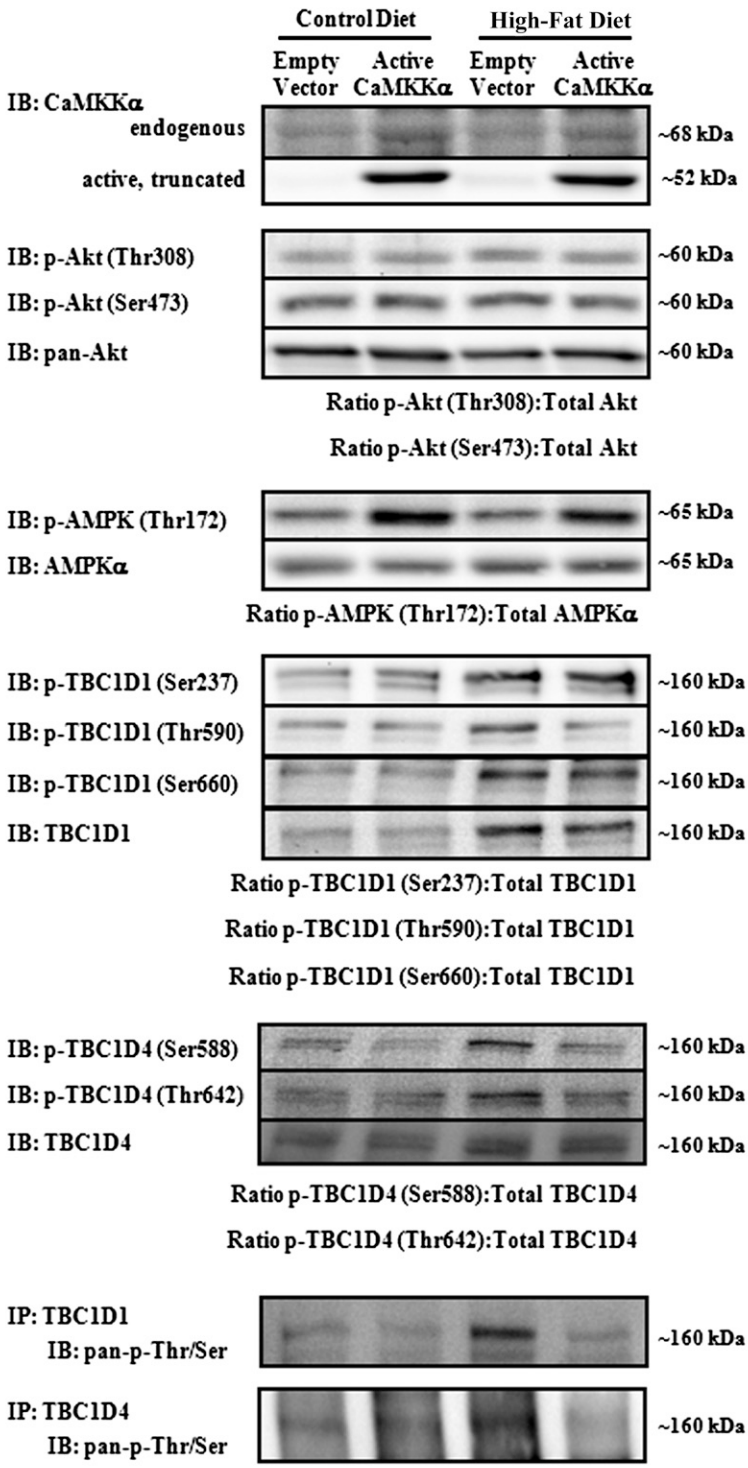
To assess whether caCaMKKα could be altering TBC1D1/TBC1D4 phosphorylation on Thr/Ser residues not examined with the site-specific phospho-antibodies, TBC1D1 or TBC1D4 was immunoprecipitated from muscle lysates and global Thr/Ser phosphorylation was assessed. As shown in Fig. 4, in control muscles caCaMKKα did not alter TBC1D1 or TBC1D4 (pan-Thr/Ser) phosphorylation. In the insulin-resistant muscles, pan-Thr/Ser phosphorylation was significantly increased on TBC1D1, and there was a tendency for it to be increased on TBC1D4 (P = 0.08); caCaMKKα attenuated those increases. These results are consistent with those obtained using the site-specific phospho-antibodies for Akt-dependent sites, but not AMPK-dependent sites.

In a Cell-Free Assay, CaMKKα Can Phosphorylate TBC1D1 and TBC1D4

In insulin-resistant muscle, caCaMKKα alters the phosphorylation status of TBC1D1 and TBC1D4. To test whether this could be occurring via direct phosphorylation of TBC1D1/TBC1D4 by CaMKKα, cell-free phosphorylation assays were performed. As shown in Fig. 5*A*, in the presence of CaMKKα and Ca^{2+}/calmodulin there was a significant increase in TBC1D1 and TBC1D4 (pan-Thr/Ser) phosphorylation, demonstrating that CaMKKα can function as an upstream kinase for TBC1D1 and TBC1D4. To examine whether this increase in pan-Thr/Ser phosphorylation could be occurring via phosphorylation of Akt- or AMPK-dependent sites, the experiments were repeated using the TBC1D1/TBC1D4 site-specific phospho-antibodies. As shown in Fig. 5*B* and *C*, in the cell-free assay CaMKKα stimulated phosphorylation of TBC1D1 (Thr^{590}) and TBC1D4 (Ser^{588}), but not TBC1D1 (Ser^{237}), TBC1D1 (Ser^{660}), or TBC1D4 (Thr^{642}), demonstrating that CaMKKα can phosphorylate TBC1D1 and TBC1D4 on some Akt- but not AMPK-specific sites.

caCaMKKα Does Not Increase GLUT1, GLUT4, or Hexokinase II Protein Levels

To evaluate the possibility that in insulin-resistant muscles caCaMKKα stimulates glucose uptake by increasing the expression of proteins involved in the transport or phosphorylation of glucose, immunoblot analyses were performed to examine GLUT1, GLUT4, and hexokinase II. Expression of caCaMKKα did not significantly alter GLUT1, GLUT4, or hexokinase II protein levels (Fig. 5), demonstrating that increases in the expression of these proteins does not account for the ability of CaMKKα to regulate glucose uptake.



Immunoblot Band Intensity (Treatment / Control, Empty Vector)

		Control Diet		High-Fat Diet	
		Empty Vector	Active CaMKKα	Empty Vector	Active CaMKKα
IB: CaMKKα endogenous	~68 kDa	1.00 ± 0.15	1.42 ± 0.14 #	1.11 ± 0.14	1.43 ± 0.16
IB: CaMKKα active, truncated	~52 kDa		1.00 ± 0.11		0.89 ± 0.11
IB: p-Akt (Thr308)	~60 kDa	1.00 ± 0.11	1.20 ± 0.12	1.16 ± 0.11	1.35 ± 0.12
IB: p-Akt (Ser473)	~60 kDa	1.00 ± 0.12	1.04 ± 0.06	1.04 ± 0.10	1.11 ± 0.09
IB: pan-Akt	~60 kDa	1.00 ± 0.07	0.92 ± 0.08	0.84 ± 0.07	0.96 ± 0.08
Ratio p-Akt (Thr308):Total Akt		1.00 ± 0.12	1.20 ± 0.19	1.40 ± 0.14 ^	1.43 ± 0.16 ^
Ratio p-Akt (Ser473):Total Akt		1.00 ± 0.16	1.08 ± 0.14	1.22 ± 0.10	1.12 ± 0.07
IB: p-AMPK (Thr172)	~65 kDa	1.00 ± 0.10	1.89 ± 0.21 #	1.04 ± 0.08	2.04 ± 0.21 #
IB: AMPKα	~65 kDa	1.00 ± 0.04	1.01 ± 0.03	1.04 ± 0.03	1.05 ± 0.05
Ratio p-AMPK (Thr172):Total AMPKα		1.00 ± 0.10	1.79 ± 0.22 #	1.00 ± 0.11	2.00 ± 0.32 #
IB: p-TBC1D1 (Ser237)	~160 kDa	1.00 ± 0.13	1.12 ± 0.15	1.58 ± 0.18 *	1.79 ± 0.35 *
IB: p-TBC1D1 (Thr590)	~160 kDa	1.00 ± 0.15	0.98 ± 0.09	2.36 ± 0.37 *	1.23 ± 0.20 #
IB: p-TBC1D1 (Ser660)	~160 kDa	1.00 ± 0.17	1.02 ± 0.13	1.75 ± 0.12 *	1.48 ± 0.19 *
IB: TBC1D1	~160 kDa	1.00 ± 0.13	0.91 ± 0.07	1.49 ± 0.12 *	1.24 ± 0.24
Ratio p-TBC1D1 (Ser237):Total TBC1D1		1.00 ± 0.16	1.13 ± 0.15	1.04 ± 0.18	1.35 ± 0.22
Ratio p-TBC1D1 (Thr590):Total TBC1D1		1.00 ± 0.12	0.97 ± 0.04	1.45 ± 0.15 *	1.07 ± 0.10 #
Ratio p-TBC1D1 (Ser660):Total TBC1D1		1.00 ± 0.07	1.16 ± 0.10	1.23 ± 0.10 ^	1.40 ± 0.17 ^
IB: p-TBC1D4 (Ser588)	~160 kDa	1.00 ± 0.14	0.85 ± 0.07	1.47 ± 0.17 *	1.13 ± 0.13
IB: p-TBC1D4 (Thr642)	~160 kDa	1.00 ± 0.19	0.86 ± 0.14	1.41 ± 0.19	0.91 ± 0.13
IB: TBC1D4	~160 kDa	1.00 ± 0.11	1.05 ± 0.14	1.20 ± 0.16	0.97 ± 0.16
Ratio p-TBC1D4 (Ser588):Total TBC1D4		1.00 ± 0.17	0.85 ± 0.13	1.39 ± 0.25	1.10 ± 0.22
Ratio p-TBC1D4 (Thr642):Total TBC1D4		1.00 ± 0.22	0.84 ± 0.17	1.29 ± 0.26	0.86 ± 0.14
IP: TBC1D1 IB: pan-p-Thr/Ser	~160 kDa	1.00 ± 0.24	0.79 ± 0.20	1.77 ± 0.25 *^	1.05 ± 0.22 #^
IP: TBC1D4 IB: pan-p-Thr/Ser	~160 kDa	1.00 ± 0.09	1.16 ± 0.08	1.71 ± 0.29	1.26 ± 0.32

Figure 4—Active CaMKKα does not stimulate muscle glucose uptake via stimulation of Akt, Akt-dependent, or AMPK-dependent phosphorylation on TBC1D1 or TBC1D4. Tibialis anterior muscles from mice fed either a control diet or a high-fat diet for 12 weeks were transfected with DNA vectors containing empty vector or caCaMKKα. After 2 weeks, muscles were excised and processed for immunoprecipitation and/or immunoblot analyses. Representative immunoblots and quantification for the phosphorylation or expression of CaMKKα, AMPK, Akt, TBC1D1, and TBC1D4. Statistical significance was defined as $P < 0.05$: *vs. control diet; #vs. empty vector; ^denotes a main treatment vs. control diet. n = 7–10 muscles per treatment group. IB, immunoblot; IP, immunoprecipitation.

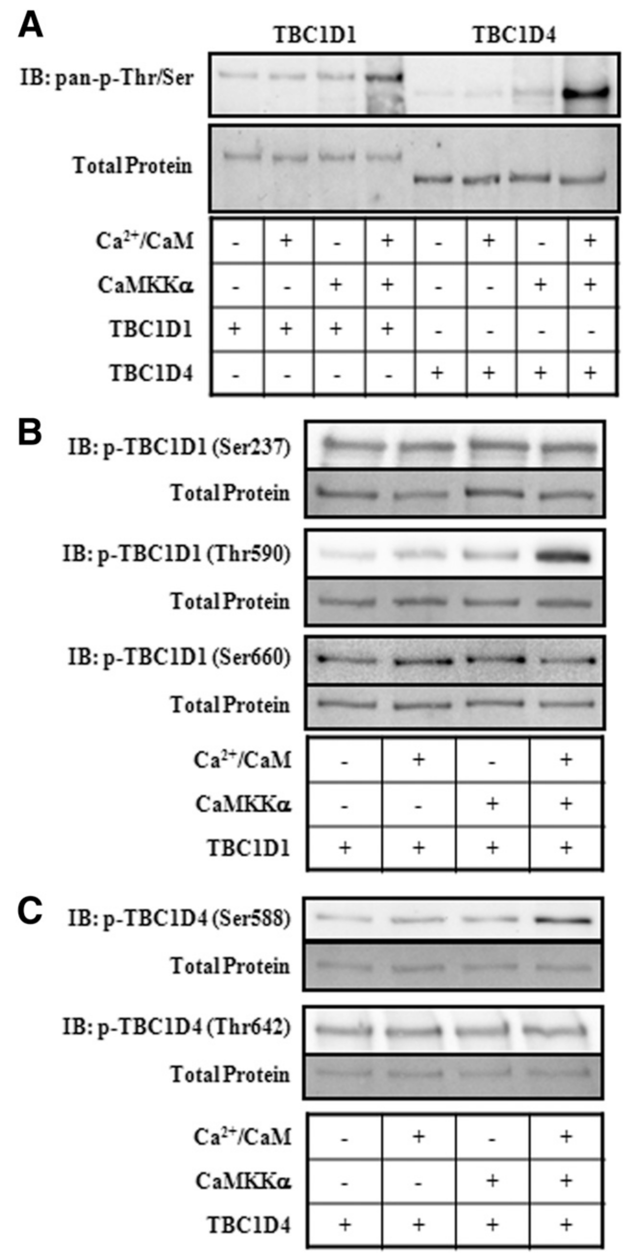


Figure 5—CaMKKα phosphorylates TBC1D1 and TBC1D4 in a cell-free assay. Recombinant, full-length, Myc-tagged TBC1D1 and TBC1D4 proteins (0.3 μg) were incubated in the presence or absence of ~3 mmol/L free Ca^{2+} plus 7 μmol/L calmodulin (Ca^{2+}/CaM) and/or recombinant GST-tagged CaMKKα isoform a (0.1 μg). *A*: TBC1D1 and TBC1D4 phosphorylation using a pan-phospho-Thr/Ser antibody. *B*: TBC1D1 phosphorylation on Ser237, Thr590, and Ser660. *C*: TBC1D4 phosphorylation on Ser588 and Thr642. Representative immunoblots and membrane stains for total protein from three independent experiments. IB, immunoblot.

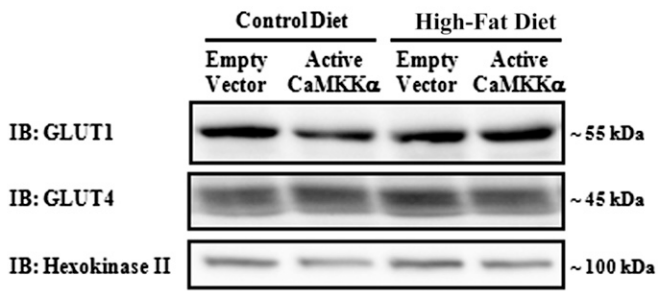
DISCUSSION

The data presented in this study are the first to demonstrate that the expression of caCaMKKα in mouse muscle can increase glucose uptake in a manner additive with insulin in insulin-sensitive muscles, and in the basal state in insulin-resistant muscles. In addition, although caCaMKKα expression does not increase TBC1D1 or TBC1D4 phosphorylation on Akt- or AMPK-dependent sites in muscle, in cell-free assays CaMKKα can function as a TBC1D1/TBC1D4 kinase, raising the possibility that CaMKKα-mediated TBC1D1/TBC1D4 phosphorylation on unique sites could be part of the mechanism underlying CaMKKα-induced muscle glucose uptake.

In this study, a high level of caCaMKKα protein expression was achieved relative to endogenous protein levels (Figs. 1*D*, 3*D*, and 4, and Supplementary Fig. 1). The physiological relevance of this high expression is difficult to assess because a truncated form of CaMKKα that would not normally be found in a mammalian cell was used. However, a study by McGee et al. (15) demonstrated that in mouse plantaris muscle 1 week of hypertrophic growth increased endogenous CaMKKα protein levels by approximately fourfold and CaMKKβ protein levels by ~50-fold. Thus, there is precedent for mouse muscle to experience a large increase in CaMKK protein content in response to a physiological stimulus. To assess the relationship of active CaMKKα protein levels and muscle glucose uptake, we performed a linear regression analysis. Importantly, we did not observe a significant correlation between the amount of caCaMKKα and the increase in glucose uptake (Supplementary Fig. 1). Thus, active CaMKKα protein expression across a wide range (~15- to 200-fold) does not affect the ability of the muscle to transport glucose.

Male C57BL/6J mice fed a 60 kcal% fat diet for 12 weeks exhibited significantly elevated fasted blood glucose and insulin levels, as well as reduced insulin-stimulated muscle glucose uptake (Fig. 2, Table 1, and Supplementary Table 1), consistent with numerous studies reporting hyperglycemia (16–19), hyperinsulinemia (16–19), and muscle insulin resistance (18,19) in C57BL/6 mice fed a high-fat diet. Importantly, although muscle insulin resistance and the ability of caCaMKKα to stimulate glucose uptake were not assessed in muscles from the same high-fat diet–fed mice, none of the mouse phenotypic characteristics were significantly different between the subsets (Supplementary Table 1). Thus, the level of muscle insulin resistance observed in one high-fat diet subset should be comparable to the other. Unfortunately, since the in vivo glucose uptake experiments require the systemic injection of $[^3H]$-2-deoxyglucose, we could not assess insulin-induced muscle glucose uptake in the same mice.

Insulin resistance was assessed in extensor digitorum longus muscles (Fig. 2), whereas the ability of caCaMKKα to stimulate glucose uptake was assessed in tibialis anterior muscles (Fig. 3). Unfortunately, the relatively large size (~45 mg) and irregular shape of the tibialis anterior prohibits its use in ex vivo studies because of limited oxygen diffusion to the interior fibers. The extensor digitorum longus is a relatively small (~10 mg), cylindrically shaped muscle and shares a similar fiber-type composition to the tibialis anterior (~88% vs. 93% type



Immunoblot Band Intensity (Treatment / Control, Empty Vector)	Control Diet: Empty Vector	Control Diet: Active CaMKKα	High-Fat Diet: Empty Vector	High-Fat Diet: Active CaMKKα
IB: GLUT1	1.00 ± 0.11	0.90 ± 0.14	0.94 ± 0.10	0.89 ± 0.12
IB: GLUT4	1.00 ± 0.19	0.95 ± 0.16	1.29 ± 0.25	1.36 ± 0.21
IB: Hexokinase II	1.00 ± 0.08	0.89 ± 0.06	0.93 ± 0.06	0.88 ± 0.10

Figure 6—Expression of caCaMKKα in mouse skeletal muscle does not increase the protein expression of GLUT1, GLUT4, or hexokinase II. Representative immunoblots and quantification (n = 8–10 muscles per treatment group). IB, immunoblot.

IIB plus type IIDB fibers, respectively) (20). Thus, insulin resistance observed in the extensor digitorum longus should be comparable to that in the tibialis anterior.

Despite an approximately twofold increase in glucose uptake in muscles expressing caCaMKKα, we did not observe a significant lowering of blood glucose or insulin levels in the insulin-resistant mice (Supplementary Table 2). This result was not unexpected because a single tibialis anterior muscle (~60 mg) accounts for only ~0.3% of the total body mass of a 20-week-old, chow-fed, male C57BL/6J mouse (average body weight in this study ~26 g).

caCaMKKα increased glucose uptake to the same extent in both insulin-sensitive and insulin-resistant muscle (Fig. 3), suggesting that CaMKKα signaling stimulates glucose uptake via an insulin-independent signaling pathway. Our results are consistent with data obtained in isolated mouse muscles, which demonstrated that inhibition of CaMKK with the chemical inhibitor STO-609 significantly inhibited caffeine-induced (6) and contraction-induced glucose uptake (7,8), but not insulin-induced glucose uptake (7).

In both insulin-sensitive and insulin-resistant muscles, caCaMKKα did not increase Akt phosphorylation or protein levels (Fig. 4). These results were consistent with previous work that demonstrated no change in Akt (Thr^{308}) phosphorylation or Akt1/2 protein levels in response to active CaMKKα expression (7). Thus, insulin resistance had no effect on the ability/inability of CaMKKα to phosphorylate Akt. These results were not consistent with previous cell-free or cell culture studies that showed that CaMKKα can phosphorylate Akt (Thr^{308}) (21). The reason behind the lack of CaMKKα-Akt signaling in muscle is not known, although it could be due to tissue-specific differences in CaMKKα function, subcellular localization, and/or lack of necessary cofactors.

Previous work has shown that insulin stimulates phosphorylation of TBC1D1 on Thr^{253} and Thr^{590} (22,23); and of TBC1D4 on Ser^{318}, Ser^{588}, Thr^{642}, Ser^{711}, and Ser^{751} (24,25). Whereas phospho-antibodies for TBC1D1 (Thr^{253}), TBC1D4 (Ser^{711}), and TBC1D4 (Ser^{751}) are currently unavailable, we were able to examine TBC1D1 (Thr^{590}), TBC1D4 (Ser^{588}), and (Thr^{642}) phosphorylation. In insulin-sensitive and insulin-resistant muscle, caCaMKKα did not increase TBC1D1 (Thr^{590}), TBC1D4 (Ser^{588}), or (Thr^{642}) phosphorylation (Fig. 4), consistent with data showing that activation of CaMKKα signaling regulates glucose uptake via an insulin/Akt-independent pathway. Intriguingly, in insulin-resistant muscle, caCaMKKα significantly decreased TBC1D1 (Thr^{590}) and attenuated TBC1D4 (Ser^{588}) phosphorylation (Fig. 4), raising the possibility that active CaMKKα inhibits this phosphorylation by stimulating phosphorylation on another residue. Consistent with this hypothesis, in cell-free assays CaMKKα can phosphorylate TBC1D1 and TBC1D4 on Thr/Ser residues (Fig. 5). This result was initially surprising since previous cell-free studies had demonstrated that CaMKKβ is not able to phosphorylate TBC1D1/TBC1D4 (PAS) (26). However, there are several possible explanations for the difference in these findings including different CaMKK isoforms (CaMKKα vs. CaMKKβ), assay conditions (calmodulin vs. no calmodulin), as well as the primary antibody (pan-Thr/Ser vs. PAS antibody). Importantly, the results from our cell-free assays are not evidence that CaMKKα phosphorylates TBC1D1/TBC1D4 in muscle, and additional studies are still needed to determine the potential significance of this interaction for muscle glucose uptake.

In control and insulin-resistant muscle, caCaMKKα increased glucose uptake independent of changes in GLUT1, GLUT4, or hexokinase II protein expression (Fig. 6). Importantly, these data do not account for possible relocalization of glucose transporters to the membrane, as this could also lead to increases in glucose uptake. Determination of the GLUTs that are mediating CaMKKα-induced glucose uptake is a topic of future investigation.

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Author Contributions. J.M.H., J.L.F., J.J.B., C.A.S.S., and L.A.A.G. participated in data collection and generation and reviewed and edited the manuscript. C.A.W. designed the experiments, participated in data collection and generation, and wrote and edited the manuscript. C.A.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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