

Calpain System Regulates Muscle Mass and Glucose Transporter GLUT4 Turnover*

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Kenichi Otani[‡], Dong-Ho Han[‡], Eric L. Ford[‡], Pablo M. Garcia-Roves[‡], Honggang Ye[§],
Yukio Horikawa[¶], Graeme I. Bell[§], John O. Holloszy[‡], and Kenneth S. Polonsky^{‡**}

From the [‡]Department of Medicine, Washington University School of Medicine, St. Louis, Missouri, 63110, the [§]Departments of Biochemistry and Molecular Biology and Medicine and the Howard Hughes Medical Institute, University of Chicago, Chicago, Illinois 60637, and the [¶]Department of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, Gunma 371–8512, Japan

The experiments in this study were undertaken to determine whether inhibition of calpain activity in skeletal muscle is associated with alterations in muscle metabolism. Transgenic mice that overexpress human calpastatin, an endogenous calpain inhibitor, in skeletal muscle were produced. Compared with wild type controls, muscle calpastatin mice demonstrated normal glucose tolerance. Levels of the glucose transporter GLUT4 were increased more than 3-fold in the transgenic mice by Western blotting while mRNA levels for GLUT4 and myocyte enhancer factors, MEF 2A and MEF 2D, protein levels were decreased. We found that GLUT4 can be degraded by calpain-2, suggesting that diminished degradation is responsible for the increase in muscle GLUT4 in the calpastatin transgenic mice. Despite the increase in GLUT4, glucose transport into isolated muscles from transgenic mice was not increased in response to insulin. The expression of protein kinase B was decreased by ~60% in calpastatin transgenic muscle. This decrease could play a role in accounting for the insulin resistance relative to GLUT4 content of calpastatin transgenic muscle. The muscle weights of transgenic animals were substantially increased compared with controls. These results are consistent with the conclusion that calpain-mediated pathways play an important role in the regulation of GLUT4 degradation in muscle and in the regulation of muscle mass. Inhibition of calpain activity in muscle by overexpression of calpastatin is associated with an increase in GLUT4 protein without a proportional increase in insulin-stimulated glucose transport. These findings provide evidence for a physiological role for calpains in the regulation of muscle glucose metabolism and muscle mass.

The presence of calpains, calcium-activated proteases, in mammalian cells was first reported over 30 years ago (1). Since that time at least 14 members of the calpain family have been identified and their chemistry and biology have been exten-

sively studied (2). It has been proposed that alterations in calpain activity result in a number of disease states, including stroke (2, 3), traumatic brain injury (2, 3), Alzheimer's disease (2, 3), cataracts (3), limb-girdle muscular dystrophy (2, 3) and gastric cancer (2). Recent studies of the genetic basis of type 2 diabetes revealed that genetic variation in calpain 10 accounted for a significant component of the genetic risk for diabetes in a Mexican-American population (4). Subsequent studies have confirmed the role of calpain 10 as a diabetes susceptibility gene in some (5) but not all (6–13) populations studied.

The pathophysiological mechanism(s) whereby genetic variation in a calpain gene could lead to alterations in glucose tolerance are not known, and only a limited number of studies have examined the potential effects of alterations in calpain activity on insulin secretion or insulin action. These studies indicate that a reduction in calpain activity caused by calpain inhibitors induces a state of insulin resistance in isolated muscle strips (14). Pancreatic islets exposed to calpain inhibitors for 4–6 h demonstrated increased glucose-induced insulin secretion, whereas longer periods of exposure induced significant defects in insulin secretory responses to glucose and other secretagogues (14). These studies are limited by the fact that all experiments were conducted *in vitro*, and nonspecific effects of the calpain inhibitors, although unlikely, cannot be completely excluded. To examine the effects of inhibiting calpain activity using another *in vivo* experimental system, we produced transgenic mice that overexpress the endogenous calpain inhibitor, calpastatin.

EXPERIMENTAL PROCEDURES

Generation of MCK-hCAST Transgenic Mice—Full-length human calpastatin (hCAST)¹ corresponding to the 673-amino acid isoform described in GenBankTM accession number 1611327A was obtained using human pancreatic islet cDNA as template and amplified with forward and reverse primers 5'-TGG TGC AAC CAG CAA GTC TTC-3' and 5'-GGA TGT TCA GAG ACT CAA C-3', respectively. The PCR product was subcloned into pCR2.1-TOPO (Invitrogen) and the sequence of the insert confirmed. The insert was excised by digestion with EcoRI and cloned into the EcoRI site of the mouse muscle-specific creatine kinase (mMCK) promoter-bovine growth hormone (bGH) polyadenylation signal vector (15). The 4.28-kb mMCK-hCAST-bGH was excised from the vector by digestion with HhaI and purified by sucrose gradient centrifugation (Fig. 1). Transgenic mice were generated by microinjection of transgene DNA into the pronucleus of fertilized single-cell C57BL/6 embryos at DNX Transgenic Sciences (Princeton, NJ). Animals were housed in a room maintained at 23 °C with a fixed 12-h light-dark cycle

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¶ An investigator of the Howard Hughes Medical Institute.

** To whom correspondence should be addressed: Dept. of Medicine, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8066, St. Louis, MO 63110. Tel.: 314-362-8061; Fax: 314-362-8015; E-mail: polonsky@im.wustl.edu.

¹ The abbreviations used are: hCAST, human calpastatin; GLUT4, glucose transporter 4; MEF, myocyte enhancer factor; mMCK, mouse muscle-specific creatine kinase; EDL, extensor digitorum longus; WT, wild type; CsTg, calpastatin overexpression transgenic.

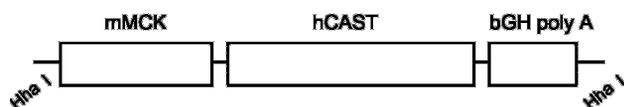


FIG. 1. Transgene construct used in the production of the MCK-hCAST transgenic (CsTg) mice.

and given free access to Purina chow and water. After an overnight fast, mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight), and the soleus, epitrochlearis, extensor digitorum longus (EDL), and other muscles were excised. All experimental procedures were approved by the Washington University Animal Study Committee.

DNA, RNA, and Protein Analysis—The mMCK-hCAST transgene was detected in transgenic mice by PCR analysis of tail DNA using forward and reverse primers 5'-GGC AAC GAG CTG AAA GCT CAT C-3' and 5'-CAG TGA TAC CAG CAA CAC TCT CTC CAC C-3'. Total RNA was isolated from skeletal muscle using TRIzol reagent (Invitrogen) and suspended in Dnase- Rnase-free distilled water (Invitrogen). Mouse GLUT4 mRNA levels were determined by competitive RT-PCR using GLUT4 forward primer plus nested forward primer 5'-TCA ATG CCC CAC AGA AGG TGT CAA TGG TTT GGA AGG AAA AGG-3' and reverse primer 5'-AAC CAG AAT GCC AAT GAC GAT G-3' as described previously (34).

Total protein extracts were prepared by homogenizing muscle in 250 mM sucrose containing 20 mM HEPES and 1 mM EDTA, pH 7.4. Protein was resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were blocked overnight at 4 °C with 5% nonfat milk in phosphate-buffered saline containing 0.1% Tween 20. The membranes were probed with the following primary antibodies: polyclonal anti-CAST (Calbiochem, La Jolla, CA), anti-GLUT1 and -GLUT4 (a generous gift from Dr. Mike Mueckler, Washington University), anti-MEF 2A (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-MEF 2D (Transduction Laboratories, Lexington, KY) as well as anti-insulin receptor β subunit, anti-insulin receptor substrate-1, anti-insulin receptor substrate-2, monoclonal anti-Akt/PKB, and anti-phosphatidylinositol 3-kinase, all from Upstate Cell Signaling Solution, Lake Placid, NY. Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), and reagents for enhanced chemiluminescence were obtained from Amersham Biosciences.

Physiological Studies—Intraperitoneal glucose tolerance tests were performed following a 4-h fast. Blood was sampled from the tail vein prior to and 30, 60, and 120 min after injection of dextrose intraperitoneally (2 g/kg body weight). For glucose transport studies, epitrochlearis, soleus, and EDL muscles were removed and allowed to recover from dissection by incubation in 2 ml of Krebs-Henseleit buffer (KHB) containing 8 mM glucose, 32 mM mannitol, and 0.1% bovine serum albumin for 1 h at 35 °C in a shaking incubator. This was followed by 30 min of incubation in the same medium with or without a maximally effective insulin concentration (2 microunits/ml). The gas phase during the incubation was 95% O₂, 5% CO₂. The muscles were then washed for 10 min in KHB containing 40 mM mannitol, 0.1% bovine serum albumin, and insulin (if it was present in the previous incubation) to remove glucose from the extracellular space. Muscles were then transferred to 1.5 ml of KHB containing 3 mM 2-deoxy-D-[1,2-³H]glucose (2DG) (1.5 μ Ci/ml), 36 mM [¹⁴C]mannitol (0.2 μ Ci/ml), 0.1% bovine serum albumin, and insulin (if it was present in the previous incubation). Extracellular space and intracellular 2DG concentration were then determined as described previously (16).

Measurement of Muscle Weight—The soleus, EDL, tibialis anterior, gastrocnemius, and quadriceps muscles were dissected out using a consistent surgical approach. The muscles were weighed immediately following dissection after blood had been removed from the muscle surface.

Measurement of Muscle Glycogen Content—Glycogen content was measured in perchloric acid extracts of muscle by the amyloglucosidase method (17).

In Vitro Cleavage of Human GLUT4 by Calpain-2—Human GLUT4 (hGLUT4) protein was prepared using a coupled *in vitro* transcription and translation system (Promega, Madison, WI). One microgram of the hGLUT4-plasmid construct pGEM4Z-AMT7 (18) was incubated in the coupled transcription and translation reaction with 20 μ Ci of [³⁵S]Met in a volume of 50 μ l to generate [³⁵S]-labeled hGLUT4. Unlabeled hGLUT4 was prepared by using 20 μ M Met in the coupled transcription and translation reaction. Four microliters of the coupled transcription

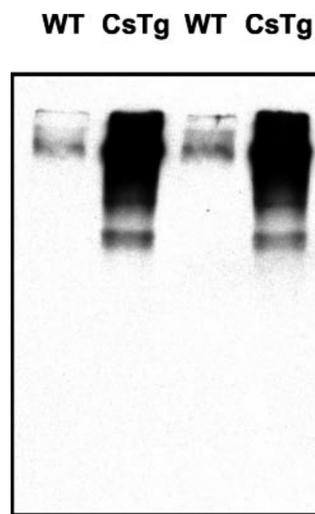


FIG. 2. Muscle calpastatin expression. Overexpression of human calpastatin in muscles of CsTg but not WT mice was confirmed by Western blot.

and translation reaction was incubated with 1.7 units of recombinant rat calpain-2 (Calbiochem) in 20 μ l of calpain cleavage buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, and 0.1% bovine serum albumin). Calpain was activated by addition of CaCl₂ to 5 mM. Cleavage was terminated after 5 and 30 min by addition of 20 μ l of SDS-sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.2 M dithiothreitol, and 0.02% bromophenol blue). Samples were heated at 55 °C for 15 min and analyzed on a 4–20% SDS-PAGE followed by autoradiography or Western blotting. The Western blot was developed using a polyclonal anti-GLUT4 antibody generated using a cytoplasmic 12-amino acid C-terminal peptide from mouse GLUT4 (Alpha Diagnostic, San Antonio, TX).

RESULTS

Generation of Transgenic Mice Overexpressing Calpastatin in Muscle—We obtained five founders (two females and three males). Human calpastatin was expressed in muscle (soleus and EDL) from all five founders. The founder used to generate the mice used in the present experiments showed a substantially greater level of calpastatin expression (~20-fold) than the other founders, which all showed a similar level of overexpression. All transgenic mice were heterozygous. All were used around 4 months of age.

Calpastatin Expression by Western Blot—Overexpression of human calpastatin in muscles was confirmed by Western blot (Fig. 2). Although human calpastatin was not expressed in muscle from WT mice, substantial expression was detected in transgenic (CsTg) mice.

Mouse Body Weight and Glucose Tolerance—The body weights of male WT mice averaged 28.1 \pm 0.6 g compared with 31.2 \pm 0.5 g for the CsTg group ($p < 0.01$, 8 mice per group, 23–25 weeks old). For the female mice, body weight averaged 21.8 \pm 0.3 g for the WT group and 23.2 \pm 0.4 g for the CsTg group ($p < 0.01$, $n = 16$ per group, 23–25 weeks old). Differences in the intraperitoneal glucose tolerance test between the WT and the CsTg mice (WT versus CsTg: 182.3 \pm 6.9 mg/dl versus 197.9 \pm 9.3 mg/dl at fasting, 189.3 \pm 7.0 mg/dl versus 198.3 \pm 10.0 mg/dl at 2 h after injection) were not statistically significant.

Insulin Action in Muscle from CsTg Mice—The increase in 2-deoxy-D-[1,2-³H] glucose transport in response to a maximally effective insulin concentration (2 microunits/ml) was not significantly different in muscles from CsTg mice as compared with the WT controls in either EDL (Fig. 3), soleus (data not shown), or epitrochlearis (data not shown). Basal rates of glucose transport were also not different.

GLUT4 Glucose Transporter Expression—As shown in Fig. 4, GLUT4 protein content of EDL, soleus, triceps, and tibialis

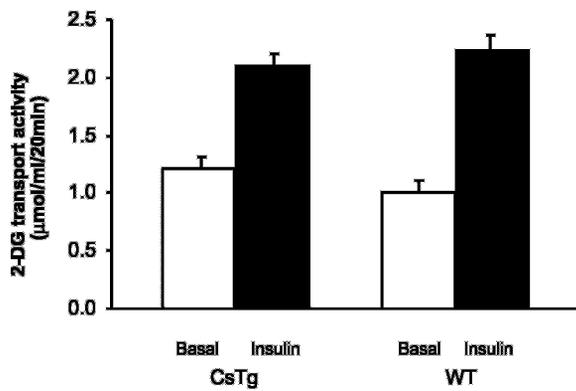


FIG. 3. **Insulin-stimulated 2-deoxyglucose transport.** EDL muscles were incubated in the absence or presence of insulin (2 microunits/ml). Values shown are means \pm S.E. for seven muscles per group. There were no significant differences between the WT and the CsTg mice.

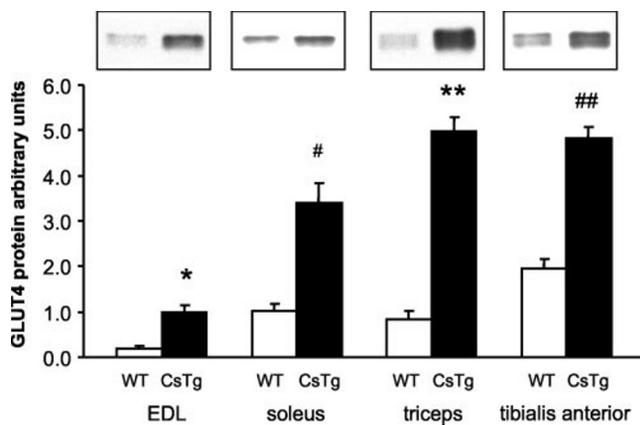


FIG. 4. **Measurement of immunoreactive GLUT4.** GLUT4 protein was determined by Western blotting in EDL, soleus, triceps, and tibialis anterior using a rabbit polyclonal antibody directed against the C terminus of GLUT4. Values are means \pm S.E. for eight muscles per group. *, $p < 0.001$; **, $p < 0.0001$; #, $p < 0.00001$; ##, $p < 0.000002$ versus WT mice.

anterior muscles was increased more than 3-fold in CsTg mice. The magnitude of insulin-stimulated muscle glucose transport is normally directly proportional to muscle GLUT4 content. This relationship is evident when muscle fiber types with different GLUT4 contents are compared (19), and in muscles that have undergone an adaptive increase in GLUT4 protein (20–22). Thus, the finding that stimulated glucose transport was the same in the CsTg as in the WT muscles despite 3-fold increases in the GLUT4 protein indicates that the increase in GLUT4 protein associated with calpastatin overexpression did not cause the expected increase in glucose transport in response to stimulation by insulin.

GLUT4 mRNA Levels—Increases in GLUT4 expression induced by various adaptive stimuli may be mediated at the transcriptional level, as evidenced by an increased GLUT4 mRNA (23). However, GLUT4 mRNA was significantly decreased in muscles from CsTg mice (Fig. 5), suggesting that the increase in GLUT4 protein was mediated by a post-transcriptional mechanism. Further support for this hypothesis is provided by the finding that MEF 2A and MEF 2D protein levels were decreased \sim 50% in the CsTg muscles. Expression of GLUT4 in striated muscle is dependent on binding of a MEF 2A-MEF 2D heterodimer to the GLUT4 promoter (24). We have found that stimuli that induce increased GLUT4 expression also result in increases in MEF 2A and MEF 2D (25). As shown in Fig. 6, A and B, both MEF 2A and MEF 2D were significantly

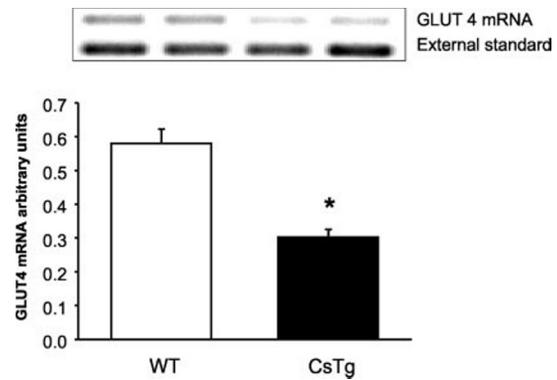


FIG. 5. **Measurement of GLUT4 mRNA.** The sequence of competitor RNA was identical to cDNA amplified by RT-PCR except for a 155-bp internal deletion (161–315). Mouse skeletal muscle total RNA was reverse-transcribed into cDNA and amplified using hybrid forward primer and reverse primer. Values are means \pm S.E. for eight muscles per group. *, $p < 0.003$.

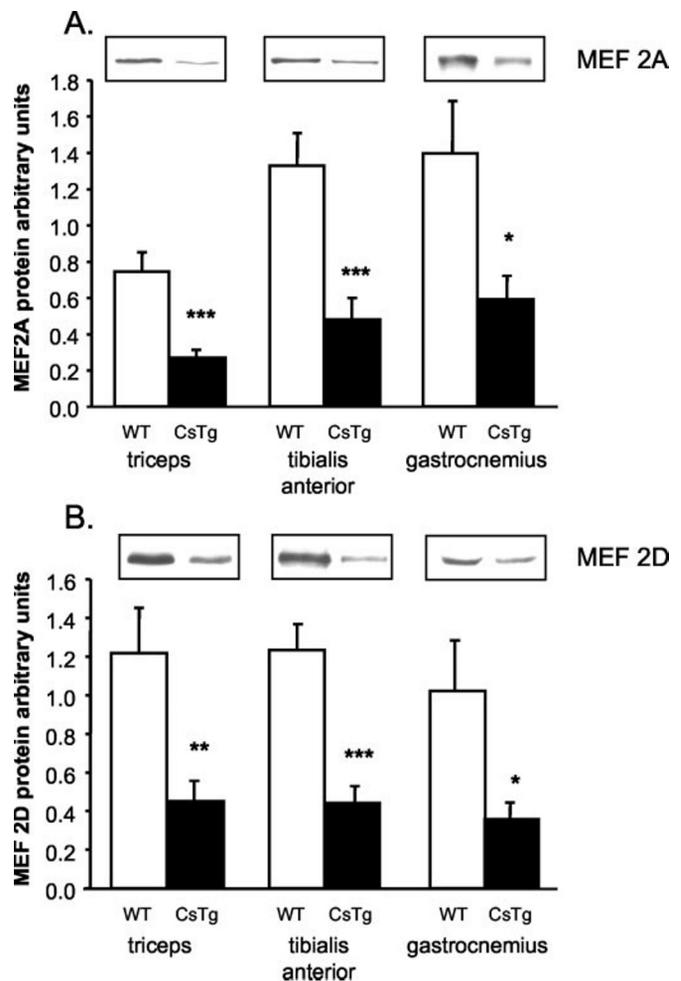
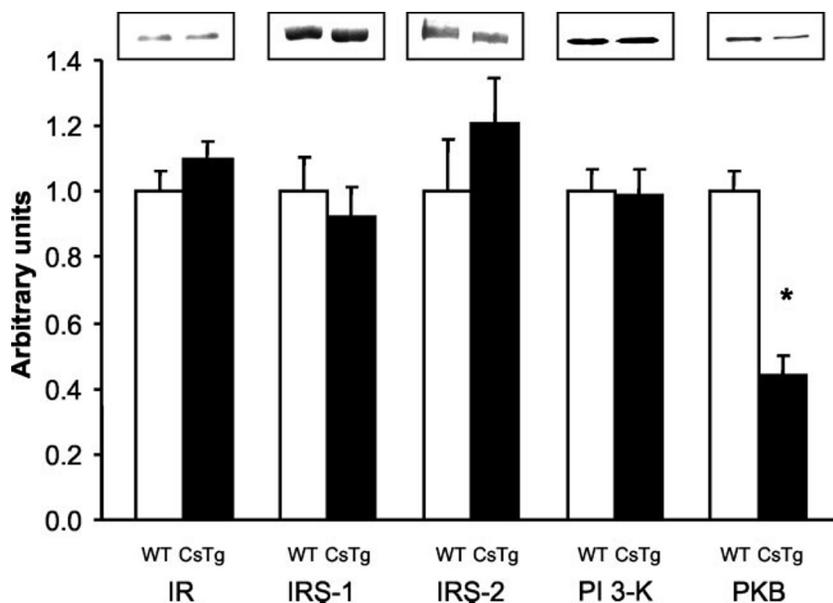


FIG. 6. **Measurement of immunoreactive MEF 2A and 2D.** A, MEF 2A. B, MEF 2D. Myocyte enhancer factors MEF 2A and 2D were measured by Western blot. Expression of GLUT4 in striated muscle is dependent on binding of MEF 2A-2D heterodimer to the GLUT4 promoter. Values are means \pm S.E. Triceps ($n = 8$), tibialis anterior ($n = 8$), and gastrocnemius ($n = 6$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

decreased in muscles from CsTg mice compared with WT controls.

GLUT1 Glucose Transporter Expression—The finding that GLUT4 protein is increased in muscles of the CsTg mice, despite a decrease in GLUT4 mRNA, led us to hypothesize that GLUT4 is a calpain substrate and that inhibition of calpain by

FIG. 7. Measurement of immunoreactive insulin receptor (IR), insulin receptor substrates (IRS) -1 and -2, protein kinase B (PKB), and phosphatidylinositol 3-kinase (PI 3-K). Values are means \pm S.E. ($n = 6$, gastrocnemius muscle); *, $p < 0.0006$.



calpastatin is responsible for the increase in GLUT4. If this hypothesis is correct, one might also expect to see an increase in GLUT1 protein because its amino acid sequence is similar to that of GLUT4 (26). We, therefore, measured the GLUT1 protein level and found that it was also increased in skeletal muscle of the CsTg mice. Although highly significant (controls, 1.04 ± 0.14 versus CsTg, 1.66 ± 0.14 arbitrary units, mean \pm S.E. for 16 muscles per group, $p < 0.004$), the magnitude of the increase was smaller than that of GLUT4.

Muscle Glycogen—Glucose transport is the primary rate-limiting step that determines the rate and extent of muscle glycogen accumulation (27). Increases in muscle GLUT4 are, therefore, generally associated with an increase in muscle glycogen content (20–22, 28, 29). Muscle glycogen concentrations were significantly increased in muscles of the CsTg mice, 20.4 ± 0.9 $\mu\text{mol/g}$ EDL WT muscle compared with 46.0 ± 2.7 $\mu\text{mol/g}$ EDL CsTg muscle.

Effect of Muscle Glycogen Depletion on Glucose Transport—Accumulation of glycogen in muscle may induce insulin resistance. To determine whether the increase in glycogen in muscles from CsTg mice was masking an increase in muscle glucose transport in CsTg muscles, we exposed muscles to hypoxia for 80 min, a maneuver that reduces glycogen. The glucose concentration in the incubation medium for the CsTg muscles was also reduced compared with the incubations for the WT muscles (2 and 8 mM glucose, respectively). This approach markedly reduced glycogen concentrations to ~ 7 $\mu\text{mol/g}$ muscle wet weight. However, even after a reduction in muscle glycogen there were no significant differences in glucose transport induced by 2 microunits/ml insulin and hypoxia in the EDL and the soleus muscles of the CsTg compared with the WT mice (data not shown). Thus, the increase in GLUT4 resulting from calpain inhibition was not associated with a parallel increase in muscle glucose transport.

Proteins of the Insulin Signaling Pathway—As a next step in our investigation of the mechanism responsible for the relative insulin resistance of the CsTg muscles, we measured the levels of some of the proteins of the insulin signaling pathway. Expression of the insulin receptor, insulin receptor substrates 1 and 2, and phosphatidylinositol 3-kinase proteins was similar in skeletal muscle of the transgenic and wild type mice (Fig. 7). However, there was a remarkable ($\sim 60\%$) decrease in protein kinase B in skeletal muscle of the CsTg mice (Fig. 7). This finding could explain why insulin-stimulated glucose transport

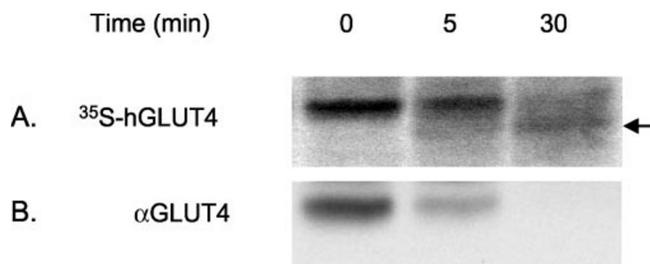


FIG. 8. Human GLUT4 is cleaved by calpain-2 *in vitro*. A, ^{35}S -labeled recombinant hGLUT4 was incubated with calpain-2 for 5 and 30 min. The samples were subjected to SDS-PAGE and autoradiography. B, unlabeled recombinant GLUT4 was incubated with calpain-2. The GLUT4 protein was identified by Western blot using an anti-C-terminal GLUT4 antibody (αGLUT4). The arrowhead indicates the GLUT4 fragment generated on incubation with calpain-2.

is not increased in CsTg muscle despite a ~ 3 -fold increase in GLUT4.

Cleavage of Human GLUT4 *In Vitro* by Calpain-2—To determine whether human GLUT4 is a calpain cleavage substrate, we incubated ^{35}S -labeled recombinant hGLUT4 with purified calpain-2. The recombinant hGLUT4 has a mass of 43.7 kDa, and incubation with activated calpain-2 generated a protein with an estimated mass of 37.2 kDa (Fig. 8A). This fragment was not identified by an antibody that binds to an epitope at the C terminus of GLUT4 (Fig. 8B), suggesting the calpain-2 cleavage site is located in the C-terminal portion of GLUT4. The mass of the cleavage product suggests that the calpain-sensitive site is about 60–70 residues from the C terminus.

Effects on Muscle Mass—To determine whether overexpression of calpastatin affected muscle mass, muscles were systematically weighed in 23–25-week-old mice. Weight of EDL and soleus muscles was increased 12% (EDL, males), 33% (EDL, females), 17% (soleus, males), and 19% (soleus, females), respectively (Fig. 9A). Similar increases in mass were present in the other muscles, including the tibialis anterior (42% in males, 47% in females), gastrocnemius (13% in males, 14% in females), and quadriceps (21% in males, 14% in females) (Fig. 9B). Total muscle protein was increased in proportion to muscle weight in EDL (WT versus CsTg: 1.96 ± 0.10 mg versus 2.40 ± 0.10 mg, $p < 0.01$) and soleus (WT versus CsTg: 1.58 ± 0.08 mg versus 1.85 ± 0.10 mg, $p < 0.05$).

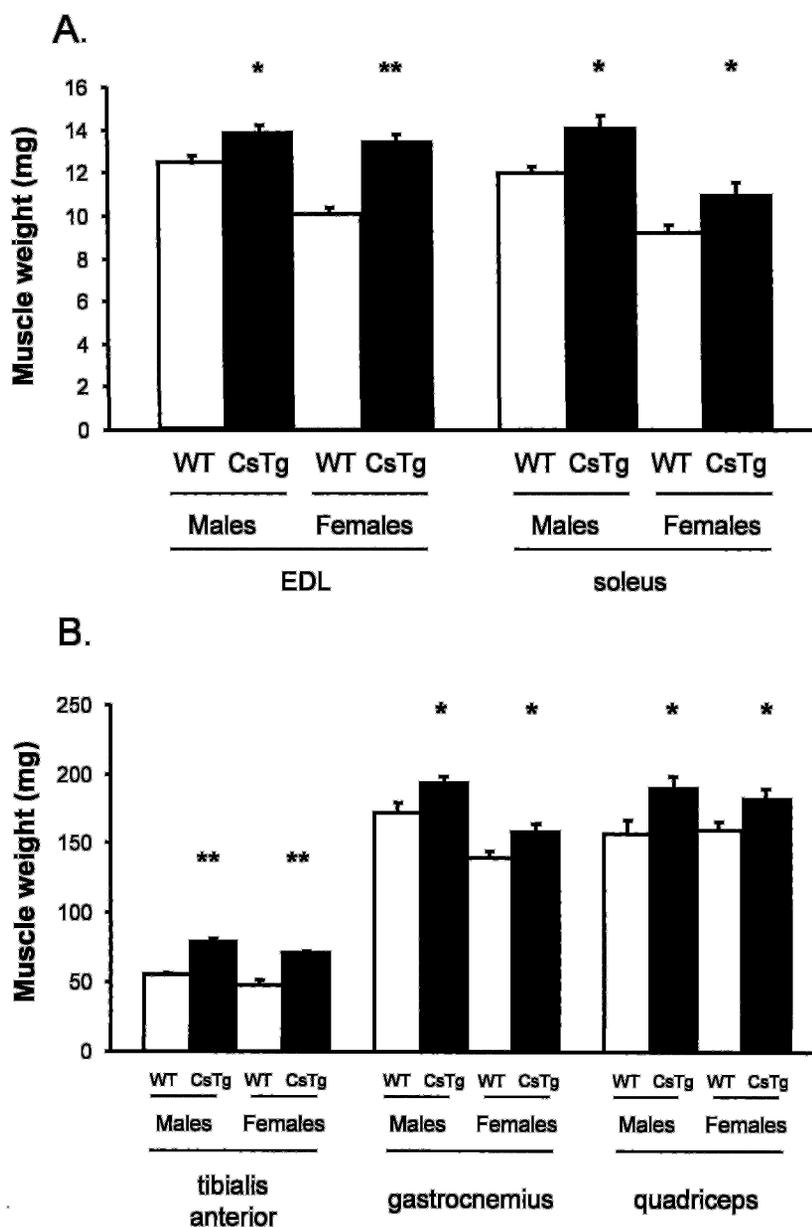


FIG. 9. **Muscle weight.** Muscles of 23–25-week-old mice were carefully dissected and weighed. Values are means \pm S.E. for six to eight muscles per group. *, $p < 0.05$; **, $p < 0.0001$.

DISCUSSION

The present study was designed to determine whether reducing calpain expression in muscle is associated with alterations in muscle glucose metabolism. A transgenic mouse with muscle-specific overexpression of calpastatin was used as the experimental model. The transgenic mice did not have overt changes in glucose tolerance and did not appear to be insulin-resistant *in vivo*. However, *in vitro* studies of muscle revealed a number of interesting effects of calpastatin overexpression.

One important change observed was a greater than 3-fold increase in GLUT4 protein detected by Western blotting. The increase was found consistently in a number of different muscles, including EDL, soleus, triceps, and tibialis anterior. It is most likely that the increase in GLUT4 is due to a reduction in the rate of GLUT4 breakdown for the following reasons. GLUT4 mRNA was not increased and was actually decreased, as were protein levels for MEF 2A and 2D, two transcription factors that mediate induction of GLUT4 expression (24). Our hypothesis is that GLUT4 serves as a calpain substrate and that the reduction in calpain activity resulting from increased expression of calpastatin leads to the increase in GLUT4 levels observed. The results of additional experiments that demon-

strated that GLUT4 is a substrate for calpain-2 *in vitro* are consistent with this idea, as is the finding that GLUT1, which has an amino acid sequence similar to that of GLUT4 (26), was also increased.

Previous experiments have shown that pharmacological inhibition of calpain causes insulin resistance of glucose transport in skeletal muscle (14). In view of this observation, we expected that overexpression of calpastatin, an endogenous calpain inhibitor, would result in severe skeletal muscle insulin resistance. Our finding that insulin-stimulated glucose transport was normal in CsTg muscles provides evidence suggesting that the greater than 3-fold increase in GLUT4 protein induced by calpastatin overexpression compensated for insulin resistance caused by inhibition of calpain activity. Maximally insulin-stimulated glucose transport is normally directly proportional to the GLUT4 protein content of a muscle (19, 21, 22). Therefore, the finding that glucose transport rates were the same in muscles of the WT and CsTg mice, despite the higher GLUT4 content of the CsTg muscles, provides evidence for a severe resistance to stimulation of glucose transport of the CsTg muscles relative to their GLUT4 content. Our finding of an ~60% reduction in protein kinase B expression in CsTg

skeletal muscle raises the possibility that an attenuation of insulin signaling via this step may play a role in mediating the relative insulin resistance. The marked decrease in expression of MEF 2A, MEF 2D, and protein kinase B provides evidence suggesting that the large increase in GLUT4 directly or by means of a secondary effect, such as the increase in glycogen, results in suppression of the expression of these proteins.

It used to be thought that calpains play a major role in muscle protein degradation only when cytosolic Ca^{2+} homeostasis is disturbed by trauma or ischemia and in muscular dystrophies (30, 31). The role of calpains in protein turnover in normal skeletal muscle is still unclear. However, evidence is accumulating that calpains mediate the initiating steps in the turnover of myofibrillar proteins. It appears that calpains are responsible for release of filaments from the myofibrils, making them available for degradation by the proteasome (32). It was recently reported that overexpression of calpastatin in skeletal muscle protects mice against the muscle atrophy associated with hindlimb unloading (33). The present finding that calpastatin overexpression results in skeletal muscle hypertrophy provides evidence that calpains are also involved in normal skeletal muscle protein turnover.

The implications of the results of this study for our understanding of the pathophysiology of type 2 diabetes are unclear. Genetic variation in a specific calpain, calpain 10, has been associated with altered risk for type 2 diabetes (4, 5). Although calpastatin inhibits the activity of calpains I and II, it has not been determined whether it has similar effects on calpain 10. Nevertheless, our results provide further evidence that inhibition of calpain activity causes insulin resistance. This finding implies that calpain plays a role in the stimulation of glucose transport. Our results also provide additional new information indicating that inhibition of calpain activity in skeletal muscle results in an increase in GLUT4 protein and causes muscle hypertrophy. These findings strongly suggest that calpains play important roles in GLUT4 and skeletal muscle proteolysis.

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