Inhibition of calpain results in impaired contraction-stimulated GLUT4 translocation in skeletal muscle

Kenichi Otani, Kenneth S. Polonsky, John O. Holloszy, and Dong-Ho Han
Department of Medicine, Washington University School of Medicine, St. Louis, Missouri
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Otani, Kenichi, Kenneth S. Polonsky, John O. Holloszy, and Dong-Ho Han. Inhibition of calpain results in impaired contraction-stimulated GLUT4 translocation in skeletal muscle. Am J Physiol Endocrinol Metab 291: E544–E548, 2006.—It was previously found that transgenic mice that overexpress the calpain inhibitor calpastatin (CsTg) have an ∼3-fold increase in GLUT4 protein in their skeletal muscles. Despite the increase in GLUT4, which appears to be due to inhibition of its proteolysis by calpain, insulin-stimulated glucose transport is not increased in CsTg muscles. PKB (Akt) protein level is reduced ∼60% in CsTg muscles, suggesting a possible mechanism for the relative insulin resistance. Muscle contractions stimulate glucose transport by a mechanism that is independent of insulin signaling. The purpose of this study was to test the hypothesis that the threefold increase in GLUT4 in CsTg would result in a large increase in contraction-stimulated glucose transport. CAMKII and AMPK mediate steps in the contraction-stimulated pathway. The protein levels of AMPK and CAMKII were increased three- to fourfold in CsTg muscles, suggesting that these proteins are also calpain substrates. Despite the large increases in GLUT4, PKB, and CAMKII, contraction-stimulated GLUT4 translocation and glucose transport were not increased above wild-type values. These findings suggest that inhibition of calpain results in impairment of a step in the GLUT4 translocation process downstream of the insulin- and contraction-signaling pathways. They also provide evidence that CAMKII and AMPK are calpain substrates.

STUDIES OF THE GENETICS of type 2 diabetes have suggested that genetic variations in calpain 10 contribute to the development of diabetes (14, 16). On the basis of this finding, the effect of calpain inhibitors on insulin-stimulated glucose transport was examined in isolated rat skeletal muscles and fat cells (15). Acute inhibition of calpain activity resulted in a large decrease in insulin-stimulated glucose transport (15). As a follow-up to this finding, we performed a study on transgenic mice that overexpress the endogenous calpain inhibitor calpastatin (CsTg) in their skeletal muscles (11). In light of the finding that pharmacological inhibition of calpain causes muscle insulin resistance (15), we expected that chronic inhibition of calpain by overexpression of calpastatin would result in severe muscle insulin resistance. Contrary to our expectation, insulin-stimulated glucose uptake in the CsTg mice, both in vivo and in muscles studied in vitro, was the same as in wild-type (WT) mice (11).

A possible explanation for this unexpected finding is that there was an approximately threefold increase in expression of the GLUT4 isoform of the glucose transporter in muscles of the CsTg mice (11). Insulin-stimulated muscle glucose transport is normally proportional to muscle GLUT4 content (6). Thus the finding that insulin-stimulated glucose transport is the same in WT and CsTg muscles is evidence that the CsTg muscles are severely insulin resistant, and that the increase in GLUT4 compensates for, i.e., corrects the insulin resistance. Protein kinase B (PKB/Akt) protein level was reduced by ∼60% in the CsTg muscles, raising the possibility that the insulin resistance of the CsTg muscles is due to the decrease in the capacity of this key step in the insulin-signaling pathway.

Glucose transport in skeletal muscle can also be activated by muscle contractions via a pathway that is independent of the insulin-signaling pathway (7). Therefore, if the decrease in insulin responsiveness relative to GLUT4 content of CsTg muscle is caused by impaired insulin signaling, it seemed possible that contraction-stimulated glucose transport might be markedly increased in CsTg muscles because of the large increase in GLUT4. The purpose of the present study was to evaluate this possibility.

MATERIALS AND METHODS

CsTg mice. CsTg mice were generated as described previously (11). The transgene DNA was targeted to skeletal muscle using the muscle-specific creatine kinase promoter.

Materials. 2-Deoxy-[1,2-3H]glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). [14C]mannitol was obtained from ICN Radiochemicals (Irvine, CA). Rabbit polyclonal anti-AMPK, anti-CAMKII (calcium/calmodulin-dependent protein kinase-II), anti-phospho-AMPK, and anti-phospho-CAMKII antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-GLUT4 antibody was a generous gift from Dr. Mike Mueckler (Washington University, St. Louis, MO). The horseradish peroxidase-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Arlington Heights, IL). The biotinylated photolabeling compound, N-[2-[(N-biotinyl-caproylamo)ethoxy]ethoxy]-4-(1-azi-2,2,2-trifluoromethyl)benzoyl-1,3-bis-(mannose-4-yloxy)-2-propylamine (biotinylated ATB-BAMPA) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). TRIZol reagent was obtained from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

Stimulation of muscles to contract. CsTg and WT mice weighing ∼25 g were provided with Purina chow and water ad libitum. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The right hindlimb was skinned and immobilized. Electrodes were attached to the sciatic nerve, and muscles were stimulated indirectly via the nerve. A Grass 548 stimulator was used to deliver 0.1-ms square-wave pulses at 100 Hz to give 250-ms-long trains at a rate of 60 trains/min for 5 min. After a
95% O2-5% CO2. The muscles were then blotted and transferred to an extracellular, i.e., [14C]mannitol, space (20).

Measurement of muscle glycogen. Soleus muscles were clamp-frozen in situ and subsequently analyzed for glycogen content. Glycogen was measured in perchloric acid extracts by the amyloglucosidase method (12).

Measurement of muscle 2-deoxyglucose transport. After dissection, soleus and EDL muscles were rinsed for 10 min in Krebs-Henseleit bicarbonate buffer (KHB), containing 40 mM mannitol, with a gas phase of 95% O2-5% CO2. The muscles were then blotted and transferred to flasks containing 1.5 ml of KHB with 4 mM 2-deoxy-[1,2-3H]glucose (2-DG, 1.5 μCi/ml) and 36 mM [14C]mannitol (0.2 μCi/ml) with a gas phase of 95% O2-5% CO2 and incubated in a shaking incubator at 29°C for 20 min. The muscles were then blotted, clamp-frozen, and processed for determination of intracellular 2-DG accumulation and extracellular, i.e., [14C]mannitol, space (20).

Measurement of muscle GLUT4 translocation. To ensure that our standard stimulation protocol, in which muscles were stimulated to contract for four 5-min intervals, the muscles were then disected. The unstimulated EDL and soleus muscles from the left hindlimb served as controls for the glucose transport and GLUT4 translocation experiments. The 2-DG transport rates shown are for extensor digitorum (EDL; A) and soleus muscles (B) from wild-type (WT) and calpastatin transgenic mice (CsTg). Values are means ± SE for 6 or 7 muscles per group.

**Western blot analysis.** Western blot analyses were performed on gastrocnemius muscle homogenates (10:1 vol/wt in RIPA buffer), as described previously for AMPK, phospho-AMPK, CAMKII, and phospho-CAMKII (18). GLUT4 was measured on soleus muscle homogenates as described previously (2).

**Semiquantitative RT-PCR.** Total RNA from gastrocnemius muscles was isolated using TRIzol reagent. DNAAse-treated total RNA was reverse transcribed (RT) into cDNA using random primer and Im Prom-II Reverse Transcriptase. Aliquots of each RT reaction were reverse transcribed (RT) into cDNA using random primer and Im Prom-II Reverse Transcriptase. Aliquots of each RT reaction were added to a PCR master mix (Promega) containing Taq DNA polymerase, dNTPs, MgCl2, reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR, and 10 pmol of sense and antisense primers. The primers used were the following: AMPKα1 forward primer 5'-AGGGCGGGGTCAATCTGAGT-3' and antisense primer 5'-AGGGCGGGGTCAATCTGAGT-3'.

**Photolabeling of muscle cell surface GLUT4.** Soleus muscles were treated as described above, but instead of measurement of 2-DG transport, the muscles were incubated in KHB buffer containing 0.5 mM biotinylated ATB-BMPA for 8 min at 18°C in the dark. Muscles were then irradiated for 2 min using a mercury vapor lamp (450 W; Conrad Hanovia); the muscles were then turned, and the other side was radiated for 2 min. Isolation of biotinylated GLUT4 was performed essentially as described by Hashimoto et al. (3). After irradiation, the muscles were homogenized in 255 mM sucrose containing 1 mM EDTA, 20 mM HEPES, 1 μg/ml each of antipain, aprotinin, pepstatin, and leupeptin, and 100 μM 4-(2-aminophenyl)benzenesulfonyl fluoride hydrochloride (AEBSF). The homogenates were centrifuged at 165,000 g at 4°C for 90 min to obtain a membrane fraction. The pellet was solubilized in Thesit detergent buffer containing (w/v) 2% Thesit, 5 mM sodium phosphate, and 5 mM EDTA, pH 7.2, and 1.0 mg/ml each of antipain, aprotinin, pepstatin, and leupeptin, and 100 μM AEBSF. The pellets were solubilized for 60 min with rotation for 60 min at 4°C followed by centrifugation at 20,000 g for 20 min at 4°C. The supernatants were treated with streptavidin beads (Pierce, Rockford, IL) to capture biotinylated proteins. The streptavidin precipitates were washed four times with PBS containing 0.1% Thesit and protease inhibitors and once with PBS. Laemmlili electrophoresis sample buffer was added to each pellet, and the samples were heated at 95°C for 30 min followed by centrifugation at 2,300 g for 1 min. This procedure was repeated, and the supernatants were combined. Mercaptoethanol was added to the supernatants to give a final concentration of 10%. The samples were then subjected to SDS-PAGE for measurement of GLUT4 protein, as described previously (2).
and reverse primer 5′-TGGGTCCTGGRGGRRRCRGRR-3′, AMPKα2 forward primer 5′-AGGGACCTGAAGCCAGAGAATG and reverse primer 5′-CTGATGCTTTGATGATCGCTCGC-3′, CAMKIIβ forward primer 5′-TGGTTTGGATTCGCGG-3′ and reverse primer 5′-CGTATGTCTTTGATAGTCGCTGCTCGC-3′, CAMKIIα forward primer 5′-TGGTTTGGTTTTGCTG-3′ and reverse primer 5′-TTGGCAGGGTTGATGGTCAG-3′, CAMKIIγ forward primer 5′-CTTGCTGGCGAGTAAATG-3′ and reverse primer 5′-TGATGGGAAATCGTAGGCTCC-3′.

The reaction mixture was subjected to PCR amplification. The lid was warmed at 94°C for 2 min, and the PCR mixtures were subjected to a 28-cycle profile that included denaturation for 30 s at 94°C, hybridization for 60 s at 58°C, and elongation for 60 s at 72°C. As an internal standard, 18S rRNA was measured simultaneously using a Quantum RNA 18S Internal Standard Kit. The PCR products were separated by electrophoresis on 2% agarose, stained with SYBER Green, photographed, and quantified by densitometry. The ratios of AMPK and CAMKII to 18S rRNA densities were then calculated.

Statistics. The results are expressed as means ± SE. The significance of difference between groups was evaluated using Student’s paired or unpaired t-test or one-way ANOVA, as appropriate. When ANOVA showed significant differences, post hoc analysis was performed with the Newman-Keuls multiple range test.

RESULTS

Muscle GLUT4 content and contraction-stimulated glucose transport activity. As in our previous study (11), soleus muscle GLUT4 content was markedly increased in the CsTg mice (3.13 ± 0.30 vs. 1.01 ± 11 arbitrary units, means ± SE for soleus muscles from 12 mice per group; P < 0.001). Stimulated muscle glucose transport normally closely parallels muscle GLUT4 content (6). However, despite the large increase in muscle GLUT4, there was no significant difference in maximal contraction-stimulated 2-DG transport between the CsTg and control groups in either the soleus or EDL muscles (Fig. 1). To ensure that the contraction stimulus was maximal, we compared the effect of our standard 10-min stimulation protocol to that of 20 min of stimulation (Fig. 1). The results were the same for the 10-min and 20-min protocols.

GLUT4 translocation to the cell surface. To determine whether the smaller increase in glucose transport activity relative to GLUT4 content in the CsTg muscles was due to impaired GLUT4 translocation, we measured cell surface GLUT4 with the biotinylated ATB-BAMPA exofacial photolabeling technique. As shown in Fig. 2, the increase in cell surface GLUT4 induced by muscle contractions was similar in the CsTg and control groups and proportional to the increase in 2-DG transport (compare Figs. 1 and 2). Thus, despite a threefold greater GLUT4 content in the CsTg muscles, the number of active GLUT4 transporters at the muscle cell surface following a maximal contraction stimulus was similar in the CsTg and control muscles.

Muscle glycogen. In contrast to muscle glycogen in the fed state, which is higher in the CsTg than in the WT mice (11), resting soleus muscle glycogen concentrations were equally low in the two groups after an overnight fast (Fig. 3). Ten minutes of contractile activity almost completely depleted muscle glycogen in both groups, and an additional 10 min of stimulation had no further effect.

AMPK and CAMKII. Activation of both AMPK and CAMKII during muscle contractions appears to be involved in mediating the increase in glucose transport induced by contractile activity (4, 5, 10, 17, 18). It therefore seemed possible...
that the resistance of GLUT4 translocation/glucose transport to stimulation by muscle contractions relative to muscle GLUT4 content might be due to downregulation of AMPK and/or CAMKII expression. However, as shown in Fig. 4, the opposite is true. AMPK protein level was increased threefold, and CAMKII protein level was increased approximately fourfold in the CsTg muscles compared with the controls. Remarkably, these large increases in AMPK and CAMKII protein levels occurred in the absence of any increase in AMPK (α1 and α2) or CAMKII (β, δ, and α) mRNA levels (Fig. 5). This finding indicates that the increases in AMPK and CAMKII proteins were mediated at the posttranscriptional level and suggest that the mechanism involved is an inhibition of proteolysis. Phosphorylated AMPK and CAMKII levels in the resting state were increased roughly in proportion to the increases in AMPK and CAMKII protein levels. However, the further increase in phosphorylation of these proteins in response to contractile activity was smaller, in relative terms, than in the control muscles (Fig. 6).

**DISCUSSION**

Overexpression of calpastatin, the endogenous inhibitor of calpain proteases, results in a more than threefold increase in the GLUT4 isoform of the glucose transporter in skeletal muscle (11). This large increase in GLUT4 protein occurs despite a decrease in GLUT4 mRNA (11). The GLUT4 protein appears to be a substrate for calpain, and it therefore seems probable that the increase in GLUT4 is mediated by decreased proteolysis as a consequence of inhibition of calpain activity by calpastatin. Despite the increase in GLUT4, insulin-stimulated glucose transport is not increased in CsTg muscle (11). Maximally stimulated glucose transport in muscle is normally proportional to muscle GLUT4 content (6, 13), so the finding of unchanged maximally insulin stimulated glucose transport, despite a large increase in GLUT4, provides evidence for severe insulin resistance relative to GLUT4 content. This finding is in keeping with the earlier observation that exposure of muscles to chemical inhibitors of calpain causes muscle insulin resistance (15).

The purpose of the present study was to determine the effect of calpastatin overexpression on contraction-stimulated glucose transport. Stimulation of muscles to contract results in an increase in glucose transport activity that is independent of, and additive to, the effect of insulin (7). The activation of both CAMKII and AMPK is involved in the stimulation of glucose transport by contractions (4, 17, 18). Although the subsequent steps in the contraction-stimulated pathway have not yet been identified, it is clear that they are separate from the insulin-signaling pathway, because inhibition of insulin signaling has no effect on stimulation of glucose transport by contractions (8, 9, 19).

Despite the approximately threefold increase in GLUT4, contraction-stimulated glucose transport activity was not increased above the WT level in the CsTg muscles. This absence of an enhancement of contraction-stimulated glucose transport is even more remarkable considering the approximately threefold increase in CAMKII and AMPK protein levels. These enzymes mediate the initial step in the contraction-signaling pathway.

**Fig. 5.** AMPK and CAMKII mRNA levels. Gastrocnemius muscle AMPK and CAMKII mRNA levels were measured using semiquantitative RT-PCR, as described under MATERIALS AND METHODS. Each bar represents the mean ± SE for 8 muscles per group.

**Fig. 6.** Phosphorylated (phospho-)AMPK and CAMKII protein levels. Phospho-AMPK (A) and phospho-CAMKII (B) were measured by Western blot analysis of unstimulated control (B) and contraction-stimulated (S) gastrocnemius muscles of WT and CsTg mice. Each bar represents the mean ± SE for 8 muscles. CsTg baseline control vs. WT baseline control, P < 0.05.
that GLUT4, AMPK, and CAMKII are calpain substrates. This is, to our knowledge, the first evidence that inhibition of calpain results in impairment of muscle GLUT4 transport.

On the basis of our previous finding that PKB/Akt protein levels were decreased by ~60% in CsTg muscles (11), we hypothesized that the lack of augmentation of insulin-stimulated glucose transport despite an approximately threefold increase in GLUT4 might be due to impaired insulin signaling. However, our finding of resistance, relative to their GLUT4 content, of CsTg muscles to contraction-stimulated transport indicates that this phenomenon is not restricted to insulin-mediated pathways but is more general and involves a mechanism common to the insulin- and contraction-stimulated pathways.

Sreenan et al. (15) have reported that treatment of muscles with a calpain inhibitor for 60 min in vitro resulted in large reductions in insulin- and hypoxia-stimulated glucose transport. There is evidence suggesting that hypoxia stimulates muscle glucose transport by the same mechanism as exercise (1). This finding of Sreenan et al. therefore supports the conclusion that inhibition of calpain results in impairment of insulin- and contraction-stimulated glucose transport. Our results provide evidence that the increase in muscle GLUT4 expression induced by chronic calpain inhibition compensates for the defect caused by calpain inhibition and thus normalizes insulin- and contraction-stimulated muscle glucose transport.

One possible mechanism relevant to both pathways is that posttranslational modification of GLUT4 made possible by a markedly reduced rate of proteolysis, i.e., an increase in 1/2, could have made the older GLUT4 molecules resistant to the signal(s) that initiates the translocation process. However, the finding that acute, pharmacological inhibition of calpain also causes insulin resistance argues strongly against this explanation. A more likely possibility is that inhibition of calpain results in impairment of one of the steps in the GLUT4 translocation process that is downstream of the insulin- and contraction-signaling pathways.

How our findings relate to the evidence that genetic variations in calpain-10 predispose to type 2 diabetes is not clear. One possibility could be that these genetic variants result in reduced calpain activity with impairment of the step that is responsible for reduced GLUT4 translocation in the CsTg muscles. Some new and unexpected findings provided by our studies on CsTg mice, that are not directly related to insulin resistance, are that inhibition of the proteolytic activity of calpain results in modest increases in skeletal muscle size (11) and large increases in GLUT4, AMPK, and CAMKII protein levels. This is, to our knowledge, the first evidence that calpains play a significant role in muscle protein turnover or that GLUT4, AMPK, and CAMKII are calpain substrates.

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GRANTS

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