Calpain facilitates GLUT4 vesicle translocation during insulin-stimulated glucose uptake in adipocytes

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Calpains are a family of non-lysosomal cysteine proteases. Recent studies have identified a member of the calpain family of proteases, calpain 10, as a putative diabetes-susceptibility gene that may be involved in the development of type 2 diabetes. Inhibition of calpain activity has been shown to reduce insulin-stimulated glucose uptake in isolated rat-muscle strips and adipocytes. In this report, we examine the mechanism by which calpain affects insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Inhibition of calpain activity resulted in approx. a 60 % decrease in insulin-stimulated glucose uptake. Furthermore, inhibition of calpain activity prevented the translocation of insulin-responsive glucose transporter 4 (GLUT4) vesicles to the plasma membrane, as demonstrated by fluorescent microscopy of whole cells and isolated plasma membranes; it did not, however, alter the total GLUT4 protein content. While inhibition of calpain did not affect the insulin-mediated proximal steps of the phosphoinositide 3-kinase pathway, it did prevent the insulin-stimulated cortical actin reorganization required for GLUT4 translocation. Specific inhibition of calpain 10 by antisense expression reduced insulin-stimulated GLUT4 translocation and actin reorganization. Based on these findings, we propose a role for calpain in the actin reorganization required for insulin-stimulated GLUT4 translocation to the plasma membrane in 3T3-L1 adipocytes. These studies identify calpain as a novel factor involved in GLUT4 vesicle trafficking and suggest a link between calpain activity and the development of type 2 diabetes.

Key words: adipocyte, actin reorganization, calpain, GLUT4, 3T3-L1, translocation.

INTRODUCTION

The identification of genetic factors involved in the development of type 2 diabetes is critical for developing effective treatments. Recent studies have identified a member of the calpain family of proteases, calpain 10 (CAPN10), as a diabetes susceptibility gene [1]. Although variations in the gene that encodes CAPN10 are associated with increased risk for type 2 diabetes in specific populations, the role of calpains in the development of type 2 diabetes is not known [2–4]. However, several lines of evidence indicate that calpains play a role in glucose homoeostasis. First, a specific single-nucleotide polymorphism-43 (SNP-43) within the CAPN10 gene, has been shown to be associated with elevated fasting blood-glucose and insulin levels and decreased CAPN10 mRNA levels in the skeletal muscle of non-diabetic Pima Indians [5]. Secondly, inhibition of calpain activity results in increased insulin secretion in isolated rat pancreatic islets [6], and thirdly, inhibition of calpain activity significantly decreases insulin-stimulated glucose uptake in isolated rat-muscle strips and adipocytes [6].

Calpains are non-lysosomal cysteine proteases that are known to cleave a wide array of protein substrates, including cytoskeletal proteins, kinases, phosphatases and transcription factors [7,8]. The physiological roles of calpain have been elucidated predominately through the use of calpain inhibitors, such as *N*-acetyl-Leu-Leu-Norleu-al (ALLN) and N-acetyl-Leu-Leu-Met-al. Calpains are involved in a number of signal-transduction pathways and have been implicated in a variety of disorders. Defects in calpain activity have been associated with the development of cataracts, age-related hypertension and type 2 diabetes [1,9,10]. In addition, our previous findings have demonstrated that calpain activity is required for 3T3-L1 pre-adipocyte proliferation and differentiation [11,12]. Taken together, these findings, as well as others, suggest that calpains function to regulate adipose-tissue mass, as well as glucose utilization [6,11,12].

Glucose uptake, which is mediated by the insulin-responsive glucose transporter 4 (GLUT4), is the rate-limiting step in glucose utilization in adipocytes. Previous studies have demonstrated that insulin-stimulated GLUT4 vesicle translocation to the plasma membrane (PM) is critical for glucose homoeostasis [13]. Expression and localization of GLUT4 are highly regulated in insulin-responsive tissues, such as skeletal muscle and adipose tissue. Insulin-stimulated glucose uptake requires the activation of several signalling pathways to mediate the translocation of GLUT4 vesicles to the PM. The binding of insulin to its receptor activates the tyrosine kinase activity of the receptor and results in autophosphorylation and the subsequent phosphorylation of insulin receptor substrate (IRS) proteins [14]. The IRS proteins initiate a cascade of events that results in the translocation of GLUT4 vesicles to the PM and glucose uptake into the cell. One of the critical targets of IRS proteins is phosphoinositide 3-kinase (PI 3-kinase), which activates downstream targets, such as the serine/threonine protein kinase Akt/protein kinase B [15]. Recent studies have implicated the phosphorylation of Akt in regulating GLUT4 translocation [16]. Another factor that has been implicated in GLUT4 translocation is dynamic actin reorganization [17–25]. Previous studies have shown that inhibition of either actin filament breakdown or formation significantly inhibited GLUT4 translocation and glucose uptake [26]. Thus, inhibition of either the insulin-stimulated PI 3-kinase pathway or the pathway

Abbreviations used: ALLN, *N*-acetyl-Leu-Leu-Norleu-al; CAPN10, calpain 10; DMEM, Dulbecco's modified Eagle's medium; 2-DOG, 2-deoxy-D-glucose; GLUT4, insulin-responsive glucose transporter 4; IRS, insulin receptor substrate; IRβ, insulin receptor β-subunit; PI 3-kinase, phosphoinositide 3-kinase; PM, plasma membrane; SNP-43, single-nucleotide polymorphism-43; TET-ON, tetracycline-regulated.

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that mediates actin reorganization results in decreased GLUT4 translocation and glucose uptake. While many factors involved in the insulin-signalling pathway have been characterized, the precise mechanism of GLUT4 translocation is poorly understood. The studies presented in this paper examine the role of calpain in glucose uptake by 3T3-L1 adipocytes, a well-characterized cell-culture model for the study of insulin-stimulated glucose uptake. Our findings demonstrate that inhibition of calpain activity reduced insulin-stimulated glucose uptake by preventing actin reorganization and thus GLUT4 translocation to the PM. More specifically, inhibition of CAPN10 protein expression decreased insulin-stimulated GLUT4 translocation and actin reorganization. We also found that calpain activity is not necessary for the proper activation of critical signalling molecules of the insulin-induced PI 3-kinase pathway.

MATERIALS AND METHODS

Materials

ALLN, leupeptin and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The anti-CAPN10 antibody was obtained from Triple Point Biologics, Inc. (Portland, OR, U.S.A.) and anti-GLUT4 goat polyclonal IgG was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Anti-insulin receptor β-subunit (IRβ) and anti-IRS-2 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-Akt and anti-phospho-Akt antibodies were obtained from New England Biolabs Inc. (Beverly, MA, U.S.A.). The anti-phosphotyrosine antibody (PY20) was obtained from BD Transduction Laboratories (Lexington, KY, U.S.A.) and the Alexa Fluor 594 donkey anti-goat IgG was from Molecular Probes, Inc. (Eugene, OR, U.S.A.).

Cell culture

3T3-L1 pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % calf serum until confluent (day -2) and maintained for an additional 2 days (until day 0). Differentiation was induced on day 0 by the addition of 0.5 mM methylisobutylxanthine, 1 μM dexamethasone, 1 μg/ml of insulin and 10 % fetal bovine serum (FBS) in DMEM. After 48 h (day 2), the medium was replaced with DMEM containing 1 μg/ml of insulin and 10 % FBS. After day 4, the cells were fed every second day with 10 % FBS in DMEM. Cytoplasmic triglyceride droplets became abundant between days 4 and 5, and by day 10, the cells were fully differentiated [27].

Glucose-uptake assay

Glucose-uptake assays were performed on day-10 3T3-L1 adipocytes. Briefly, pre-adipocytes were cultured in 6-well plates and induced to differentiate using the methylisobutylxanthine/dexamethasone/insulin protocol, as described in the previous section. Day-10 adipocytes were serum-starved for 4 h in the presence or absence of either 100 μM ALLN, 100 μM leupeptin or 100 μM pepstatin A, as indicated. Adipocytes were either untreated (basal) or treated with insulin (100 nM) for 10 min, followed by the addition of [1-^{14}C]2-deoxy-D-glucose ([^{14}C]2-DOG) (0.1μCi/well) (PerkinElmer/New England Nuclear, Shelton, CT, U.S.A.) and 5 mM glucose for an additional 10 min at 37 °C. Cells were then washed three times with ice-cold PBS and solubilized in a solution containing 0.5 M NaOH and 0.1 % (w/v) SDS. Samples were assayed for [^{14}C]2-DOG-uptake as disintegrations per min and normalized to the basal level of glucose uptake, which was set at 1.

Calpain assay

Calpain activity was determined by measuring the hydrolysis of the fluorogenic calpain substrate succinyl-leucyl-leucyl-valyl-tyrosyl-7-amino-4-methylcoumarin (Biomol, Plymouth Meeting, PA, U.S.A.) [28]. Day-10 3T3-L1 adipocytes were serum-starved for 4 h and treated according to the glucose-uptake protocol, as described above. The cells were then washed twice with ice-cold PBS containing 1 mM Na_3VO_4, and lysed in a buffer containing 50 mM Hepes (pH 7.6), 150 mM NaCl, 1 % (v/v) Triton X-100, 10 mM 2-mercaptoethanol, 0.1 mM PMSF and 10 μg/ml leupeptin. Equal volumes of lysates were diluted in Hanks balanced salt solution (Invitrogen/Gibco, Carlsbad, CA, U.S.A.) and incubated at 37 °C. At $t = 0$ min, 50 μM succinyl-leucyl-leucyl-valyl-tyrosyl-7-amino-4-methylcoumarin was added, and fluorescence was measured for 3 min at excitation and emission wavelengths of 380 nm and 460 nm, respectively. Protein concentrations were determined using the bicinchoninic acid protein determination kit (Pierce, Rockford, IL, U.S.A). To calculate the relative calpain activity, the rate of increase in fluorescence was divided by the protein concentration and normalized to basal activity, which was set at 1.

Immunofluorescence

3T3-L1 pre-adipocytes were cultured on sterilized glass coverslips and induced to differentiate using the methylisobutylxanthine/dexamethasone/insulin protocol, as described above. Day-10 adipocytes were serum-starved for 4 h and treated according to the glucose-uptake protocol (omitting [^{14}C]2-DOG). After treatment, cells were fixed with 2 % buffered paraformaldehyde, permeablized in 0.25 % (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 5 min on ice, and incubated with an anti-GLUT4 antibody or rhodamine-labelled phalloidin. Treated coverslips were mounted on glass slides and visualized by fluorescent microscopy.

Plasma lawn assay

Day-10 adipocytes were serum-starved for 4 h and treated according to the glucose-uptake protocol (omitting [^{14}C]2-DOG). A plasma lawn assay was performed as described by Kanzaki and Pessin [26]. Briefly, adipocytes were rinsed with ice-cold PBS and incubated in 0.5 mg/ml of poly-(L-lysine) for 1 min. Cells were then swollen in a hypotonic buffer (23 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.5) and sonicated in 15 ml of sonication buffer (70 mM KCl, 30 mM Hepes, 6 mM $MgCl_2$, 3 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, pH 7.5) using a probe sonicator. Plasma membrane sheets were washed twice with sonication buffer, incubated with an anti-GLUT4 antibody, and subjected to fluorescent microscopy. Images from three independent experiments (six random images from each treatment) were quantified using Image-Pro Plus software (Silver Spring, MD, U.S.A.).

Subcellular fractionation

Day-10 adipocytes were serum-starved for 4 h and treated according to the glucose-uptake protocol (omitting [^{14}C]2-DOG). Subcellular fractionation was performed as described by Piper et al. [29]. Briefly, cells were collected in HES buffer [255 mM sucrose, 20 mM Hepes (pH 7.4) and 1 mM EDTA] and homogenized with a Dounce homogenizer (seven strokes). The

homogenate was centrifuged at 19 000 ***g*** for 20 min. The resulting supernatant was centrifuged at 41 000 ***g*** for 20 min to collect high-density microsomes. Low-density microsomes were obtained by centrifugation of the previously obtained supernatant at 180 000 ***g*** for 75 min. The pellet obtained from the initial centrifugation was layered on a 1.12 M sucrose gradient and centrifuged at 100 000 ***g*** for 60 min to obtain the PM fraction and the mitochondrial/nuclear fraction. The PM fraction was then centrifuged at 40 000 ***g*** for 20 min.

Immunoprecipitation assays and immunoblotting

Cell monolayers (on 10-cm plates) were washed once with PBS and lysed in a buffer containing 25 mM Hepes (pH 7.4), 1 % Nonidet P-40, 100 mM NaCl, 2 % (v/v) glycerol, 5 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM $NaPP_i$, 1 mM PMSF, 10 mg/ml aprotinin, 5 mg/ml leupeptin and 5 mg/ml pepstatin [26]. Lysates were gently mixed for 20 min and then centrifuged for 20 min at 4 °C before determination of protein concentration by the bicinchoninic acid method. For immunoprecipitation assays, antibodies against either IRβ (10 μg/ml) or IRS-2 (8 μg/ml) were added to protein lysates (600 μg); incubation occurred for 1 h at 4 °C. Agarose beads (Protein A/G PLUS-Agarose beads, Santa Cruz Biotechnology Inc.) were mixed with the immunoprecipitates on a rocker for 1 h at 4 °C. Immunoprecipitates were recovered by centrifugation at 2500 ***g*** and washed three times with ice-cold lysis buffer. Immunoprecipitated proteins were dissolved in 2 × Laemmli buffer, heated for 5 min at 95 °C, subjected to SDS/PAGE, and transferred to Immobilon-P membranes (Millipore, Billerica, MT, U.S.A.). Membranes were incubated with the indicated primary antibodies and visualized by enhanced chemiluminescence. Protein bands were quantified by densitometry using NIH Image (v1.29) software.

Stable transfection

A tetracycline-regulated (TET-ON) expression system (Invitrogen, Carlsbad, CA, U.S.A.) was used to express a full-length antisense CAPN10 cDNA in 3T3-L1 adipocytes. Pre-confluent pre-adipocytes were co-transfected with a pBI-TET-antisense CAPN10 vector and a pRevTRE expression vector. Clonal cell lines were selected for G418 resistance. Cells harbouring the transgene were induced to differentiate for 6 days as described above. Cells were then incubated in the presence or absence of doxycycline for 72 h and subjected to the immunofluorescence protocol and probed with either an anti-GLUT4 antibody or rhodamine-labelled phalloidin.

RESULTS

Inhibition of calpain activity reduces insulin-stimulated glucose uptake in 3T3-L1 adipocytes

Previous studies have shown that inhibition of calpain activity results in a decrease in insulin-stimulated glucose uptake in rat adipocytes [6]. Since there are no CAPN10-specific chemical inhibitors available at present, Sreenan et al. [6] used a variety of protease inhibitors to implicate calpain activity in glucose homoeostasis. To investigate further the role of calpain in glucose uptake, we chose the 3T3-L1 cell line model, which has been used extensively to delineate the mechanism of insulin-stimulated glucose uptake in adipocytes. In order to determine whether 3T3-L1 adipocytes are similar to primary rat adipocytes in their requirement for calpain activity during insulin-stimulated glucose uptake, we serum-starved day-10 3T3-L1 adipocytes for 4 h in the absence or presence of the calpain inhibitor ALLN (100 μM) and performed radiolabelled [1-^{14}C]2-DOG uptake assays as described in the Materials and Methods section. As shown in Figure 1(A), [^{14}C]2-DOG uptake was increased by approx. 8-fold in insulin-stimulated 3T3-L1 adipocytes compared with unstimulated adipocytes. In 3T3-L1 adipocytes that were stimulated with insulin in the presence of ALLN, [^{14}C]2-DOG uptake was decreased by approx. 60 % compared with adipocytes stimulated with insulin alone (Figure 1A). Since calpain is a cysteine protease, we examined the effect of leupeptin, a cell-permeant cysteine-protease inhibitor [30], on insulin-stimulated

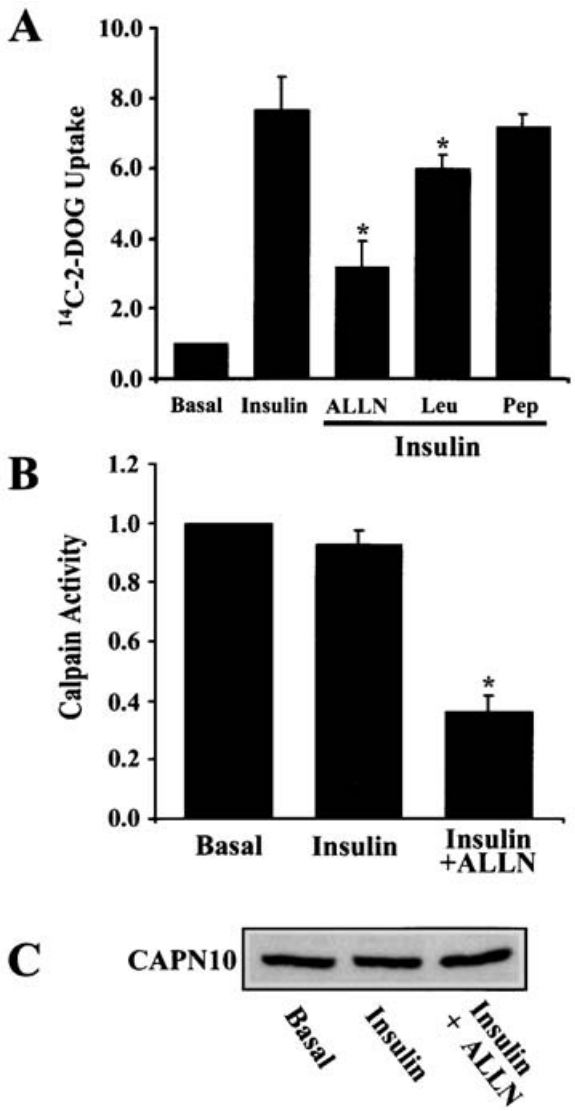

Figure 1 ALLN treatment results in decreased insulin-stimulated glucose uptake and calpain activity

Day-10 3T3-L1 adipocytes were serum-starved and incubated in the absence or presence of ALLN (100 μM) and subsequently treated with or without insulin (100 nM). (**A**) 3T3-L1 adipocytes were assayed for [^{14}C]2-DOG uptake under the basal condition or after insulin stimulation in the absence and presence of ALLN, 100 μM leupeptin (Leu) or 100 μM pepstatin A (Pep). The results are the means ± S.D. of three independent experiments. *Significantly different from insulin-treated level ($P < 0.01$). (**B**) 3T3-L1 adipocytes were assayed for relative calpain activity as described in the Materials and Methods section. The results are the means ± S.E.M. of ten assays. *$P < 0.001$). (**C**) Lysates were collected from cells with different treatments as indicated and CAPN10 protein levels were assayed by Western-blot analysis using a specific anti-CAPN10 antibody.

glucose uptake. In 3T3-L1 adipocytes treated with insulin plus leupeptin (100 μM), [^{14}C]2-DOG uptake was reduced by approx. 25 % compared with the level in adipocytes stimulated with insulin alone (Figure 1A). In contrast, the presence of the cell-permeant aspartic-protease inhibitor pepstatin A (100 μM) [31] had no significant effect on insulin-stimulated [^{14}C]2-DOG uptake (Figure 1A). The addition of protease inhibitors had no effect on glucose uptake in unstimulated adipocytes (results not shown).

To verify that calpain activity was inhibited by ALLN during insulin-stimulated glucose uptake, calpain activity was assayed in adipocytes immediately after insulin-stimulated glucose uptake by using a spectrofluorimetric assay (Figure 1B) [28]. As shown in Figure 1(B), ALLN treatment inhibited insulin-stimulated calpain activity by approx. 70 %, which correlates with the inhibitory effect of ALLN on insulin-stimulated glucose uptake (60 %) [Figures 1(A) and 1(B)]. Since CAPN10 has been implicated specifically in the development of type 2 diabetes, it was of interest to determine whether the decrease in calpain activity was due to lower levels of CAPN10 expression. As shown in Figure 1(C), the levels of CAPN10 protein were unaffected by either insulin or insulin-plus-ALLN, compared with the basal level. These results demonstrate that 3T3-L1 adipocytes are similar to primary rat adipocytes in their requirement for calpain activity during insulin-stimulated glucose uptake [6].

Calpain is required for GLUT4 vesicle translocation during insulin-stimulated glucose uptake in adipocytes

A critical event in insulin-stimulated glucose uptake is the translocation of insulin-responsive GLUT4 vesicles to the PM. Since inhibition of calpain activity decreased glucose uptake significantly, it was of interest to determine whether calpain plays a role in insulin-stimulated GLUT4 translocation. Immunofluorescent studies were conducted to determine the effect of calpain inhibition on intracellular localization of GLUT4 vesicles prior to and during insulin-stimulated glucose uptake. Day-10 3T3-L1 adipocytes were either untreated (basal), treated with insulin or treated with insulin plus ALLN, as described in the Materials and Methods section. Immunofluorescent microscopy revealed that GLUT4 vesicles were localized primarily to the perinuclear region under the basal condition (Figure 2A, panel a). However, upon insulin stimulation, there was a marked increase in GLUT4 translocation to the PM, as exhibited by the increase in fluorescent staining at the cell membrane (Figure 2A, panel b). In contrast, adipocytes treated with insulin plus ALLN showed a decreased level of GLUT4 translocation to the PM compared with insulin-stimulated adipocytes (Figure 2A, panel c).

In addition to the immunofluorescent studies, we conducted plasma lawn assays to confirm quantitatively [32–34] that calpain is required for GLUT4 translocation to the PM during insulin-stimulated glucose uptake in the absence or presence of ALLN. Under the basal condition, only a low level of GLUT4 staining was detectable in PM sheets (Figure 2A, panel d). Upon insulin stimulation, GLUT4 localization to the PM sheets increased by approx. 3-fold (Figure 2A, panel e). These results are consistent with previous studies that examined GLUT4 translocation to the PM in response to insulin stimulation [26]. In contrast, insulin stimulation in the presence of ALLN resulted in only a 1.4-fold increase in the level of GLUT4 protein present at the PM. (Figure 2A, compare panel e with panel f). To demonstrate further that calpain activity is required for insulin-stimulated GLUT4 translocation, we conducted subcellular fractionation analysis (Figure 2B). Under the basal condition, a low level of GLUT4 protein was detected in the PM fraction. Upon insulin stimulation, GLUT4 levels in the PM fraction increased by approx. 2.5-fold,

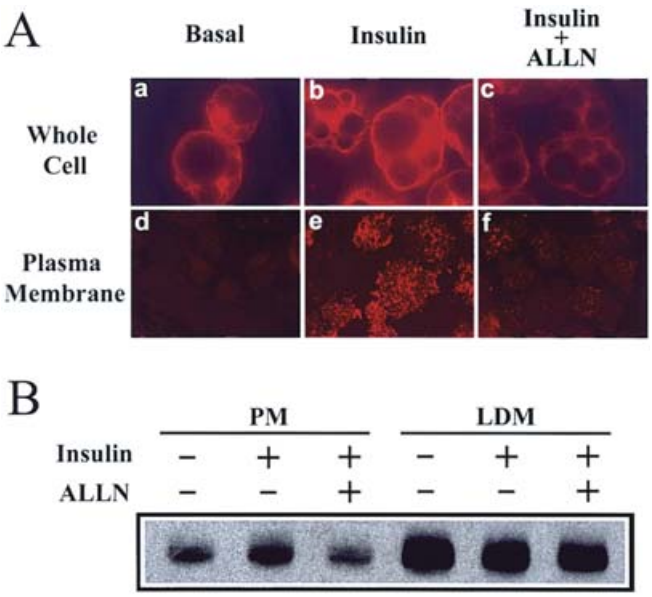

Figure 2 Insulin-stimulated GLUT4 translocation is inhibited by ALLN treatment

Day-10 3T3-L1 adipocytes were treated according to the glucose-uptake protocol as described in the Materials and Methods section, and GLUT4 protein was visualized by either immunofluorescence or subcellular fractionation followed by Western-blot analysis using an anti-GLUT4 antibody. (**A**) GLUT4 localization was assayed in whole cells (panels a–c) as well as in PM sheets (panels d–f) under the basal condition or after stimulation with insulin in the absence and presence of ALLN. Quantification of GLUT4 was determined using Image-Pro Plus software. The results are representative images from three independent experiments. (**B**) Cell homogenates were subjected to subcellular fractionation, and PM- and low-density microsome (LDM)-fractions were obtained and subjected to SDS/PAGE as described in the Materials and Methods section. GLUT4 protein levels were assayed by Western-blot analysis using an anti-GLUT4 antibody. Quantification of GLUT4 was determined using NIH Image (v1.29) software. Results are representative of three independent experiments.

whereas insulin stimulation in the presence of ALLN resulted in a 45 % decrease in the levels of GLUT4 protein in the PM fraction. These findings are consistent with the results obtained from the PM lawn analysis. We also determined whether the decrease in GLUT4 protein at the PM was due to a decrease in total GLUT4 protein levels in the cell. ALLN treatment did not affect the total GLUT4 protein level, as determined by Western-blot analysis using an anti-GLUT4 antibody (results not shown).

Calpain is not required for the expression or insulin-mediated phosphorylation of critical signalling molecules involved in GLUT4 translocation

Activation of the insulin receptor stimulates several different signalling pathways that act co-ordinately to translocate GLUT4-containing vesicles to the PM. In order to determine whether calpain affects proximal events of the insulin-signalling pathway, we examined critical signalling molecules of the insulin-induced PI 3-kinase pathway. Since calpain is a protease capable of cleaving a variety of proteins, and thus capable of affecting their activities, the protein levels and phosphorylation status of critical signalling molecules involved in insulin signalling during glucose uptake were examined. Inhibition of calpain activity by ALLN did not affect the protein levels of IRβ (Figure 3A), the proximal signalling molecule IRS-2 (Figure 3A) or Akt (Figure 3B). Since the activities of many of the signalling molecules in the insulin-signalling pathway are regulated by phosphorylation, we investigated whether calpain activity is required for the

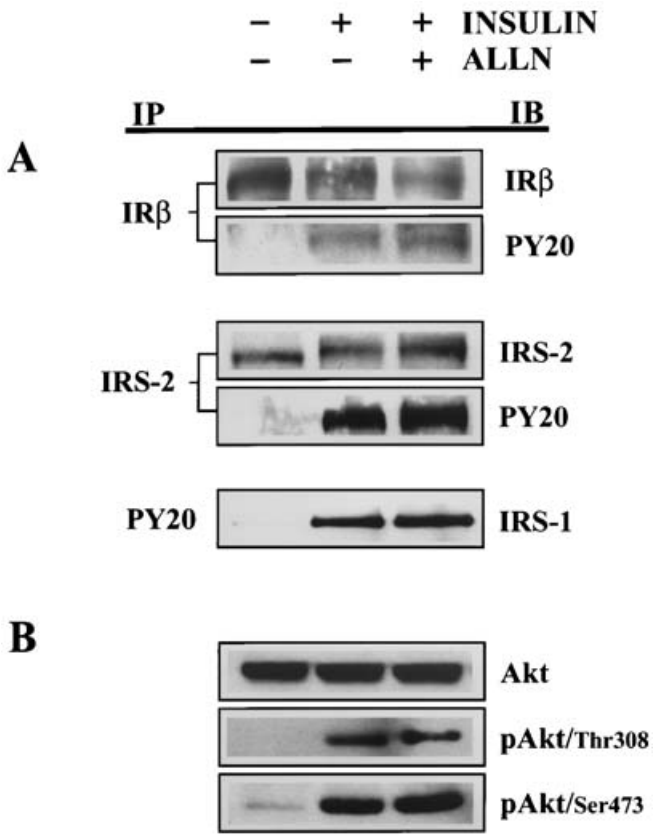

Figure 3 ALLN does not alter the expression or phosphorylation pattern of critical proteins involved in insulin-stimulated GLUT4 translocation

Day-10 3T3-L1 adipocytes were treated according to the glucose-uptake protocol in the absence and presence of ALLN (100 μM) as described in the Materials and Methods section. (**A**) IRβ and IRS-2 were immunoprecipitated (IP) from cell lysates, analysed by SDS/PAGE, and then immunoblotted (IB) with anti-IRβ or anti-IRS-2 antibodies, as indicated. The membranes were stripped and reprobed with an anti-phosphotyrosine antibody (PY20). For IRS-1, tyrosine-phosphorylated proteins were immunoprecipitated with PY20 and then blotted with anti-IRS-1 antibodies. (**B**) Cell lysates were subjected to SDS/PAGE and then immunoblotted using specific antibodies that recognized either Akt or phosphorylated Akt at Thr^{308} (pAkt/Thr308) or Ser^{473} (pAkt/Ser473). The results are representative of three independent experiments.

insulin-mediated phosphorylation of these proteins. Inhibition of calpain by ALLN treatment for 4 h (a time period that inhibited insulin-stimulated glucose uptake) did not alter the insulin-stimulated autophosphorylation of immunoprecipitated IRβ, or the phosphorylation status of IRS-1 or IRS-2 (Figure 3A). We also examined the phosphorylation status of the distal signalling molecule Akt. Inhibition of calpain by ALLN did not alter the insulin-stimulated phosphorylation of Akt at either Thr^{308} or Ser^{473} in 3T3-L1 adipocytes (Figure 3B). Although the phosphorylation patterns of three critical signalling molecules were unaffected by calpain inhibition, our findings do not exclude the possibility that calpain might alter a critical step that is downstream of Akt, but upstream of vesicle translocation or other insulin-stimulated pathways.

Inhibition of calpain activity blocks insulin-induced actin-filament reorganization

Actin reorganization has been implicated in GLUT4 vesicle trafficking [17,19,21–23,35,36]. In adipocytes, filamentous actin is localized to the cell cortex. Previous studies have shown that disruption of cortical actin reorganization by either latrunculin

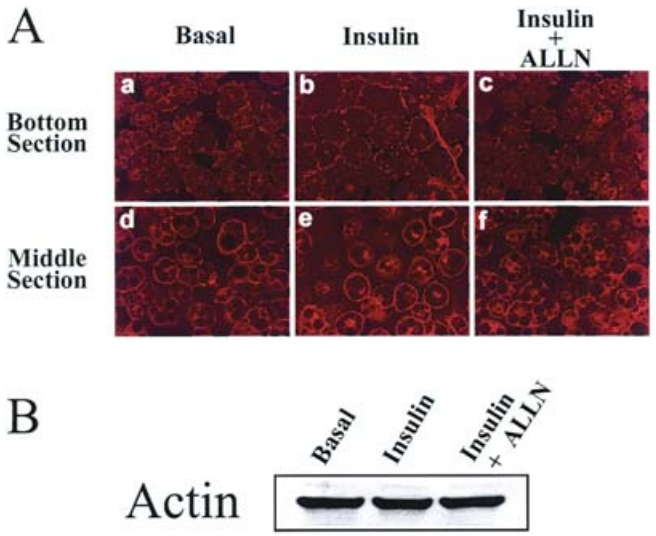

Figure 4 Inhibition of calpain activity alters cortical actin reorganization

(**A**) Day-10 3T3-L1 adipocytes were treated according to the glucose-uptake protocol as described in the Materials and Methods section, and actin filaments were visualized by rhodamine-labelled phalloidin immunofluorescence. Actin-filament localization and integrity was assayed in whole cells under the basal condition (panel a and d), after stimulation with insulin (panel b and e) or in the presence of insulin and ALLN (panel c and f). Results are representative images from three independent experiments. (**B**) Total protein lysates were collected from cells with different treatments, as indicated, and subjected to SDS/PAGE. Total actin protein levels were assayed by Western-blot analysis using an actin-specific antibody.

B (an actin-monomer-sequestering agent) or jasplakinolide (an agent that stabilizes actin filaments) inhibited insulin-stimulated GLUT4 translocation to the PM and subsequent glucose uptake [26]. Since calpains have been implicated in cytoskeletal reorganization during cell migration and cell adhesion [28,37–42], we examined the effect of decreased calpain activity on actin reorganization during insulin-stimulated glucose uptake. Adipocytes were grown on coverslips, serum-starved for 4 h in the absence or presence of ALLN and then subjected to the glucose-uptake protocol as described in the Materials and Methods section. The presence of actin filaments was assayed using rhodamine-labelled phalloidin and immunofluorescence. As shown in Figure 4(A), unstimulated adipocytes have a diffuse actin-staining pattern (Figure 4A, panels a and d). Upon insulin-stimulation, cortical actin is reorganized and increased filamentous actin staining is detected adjacent to the cortical region and the perinuclear region of the cell (Figure 4A, panels b and e). In the presence of insulin plus ALLN, there is significantly less filamentous actin staining at the cortex and the perinuclear region when compared with insulin-stimulated cells (Figure 4A, panels c and f). In order to determine whether the decrease in filamentous actin staining in the presence of ALLN was due to decreased levels of total actin protein, we performed Western-blot analysis using an anti-actin antibody (Figure 4B). Similar levels of total actin protein were detected in the absence or presence of ALLN. These findings suggest that inhibition of calpain activity blocked actin-filament formation.

Inhibition of CAPN10 decreases insulin-stimulated GLUT4 translocation and actin reorganization

While previous studies that investigated the role of calpain in glucose uptake have utilized various calpain inhibitors, the specific role of CAPN10 has not been examined. In order to investigate

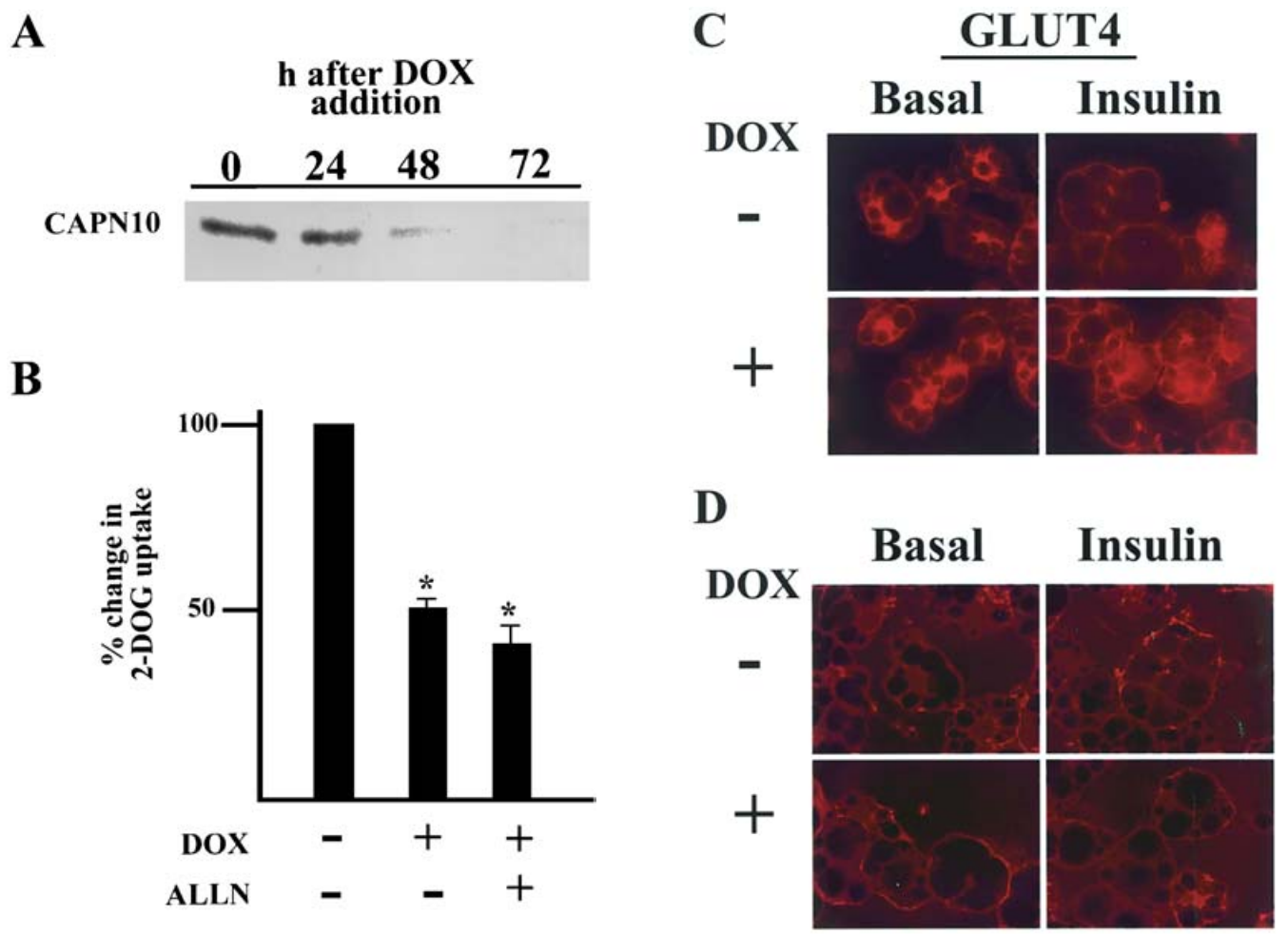

Figure 5 Inhibition of CAPN10 decreases insulin-stimulated GLUT4 translocation and actin reorganization

Day-6 3T3-L1 cells that were stably transfected with a CAPN10 antisense expression vector were incubated in the presence or absence of doxycycline (DOX) for various times. (**A**) Total protein lysates were collected from cells at the various time points, as indicated, and subjected to SDS/PAGE. Total CAPN10 protein levels were assayed by Western-blot analysis using a CAPN10-specific antibody. Day-6 3T3-L1 adipocytes harbouring the transgene were treated for 72 h in the presence (+ DOX) or absence (− DOX) of DOX and then subjected to the glucose-uptake protocol as described in the Materials and Methods section in the absence and presence of 100 μM ALLN (**B**). The results are the means ± S.D. of three independent experiments. *Significantly different from insulin-treated level, $P < 0.01$. (**C**) GLUT4 protein was visualized by immunofluorescence using a GLUT4-specific antibody and (**D**) actin filaments were visualized by immunofluorescence using rhodamine-labelled phalloidin. The results are representative of three independent experiments.

the role of CAPN10 in glucose uptake, we generated 3T3-L1 cell lines that were stably transfected with a TET-ON CAPN10 antisense expression vector. A representative cell line harbouring the CAPN10 antisense expression vector was induced to differentiate for 6 days and then incubated in the presence of doxycycline for various times to determine the time required to reduce CAPN10 expression. As shown in Figure 5(A), there was minimal CAPN10 expression 72 h after addition of doxycycline to the culture medium. Based on this finding, we conducted our experiments 72 h after doxycycline treatment. To determine whether CAPN10 plays a role in insulin-stimulated glucose uptake, we stimulated 3T3-L1 adipocytes harbouring the transgene with insulin in the presence or absence of doxycycline and then performed a glucose-uptake assay (Figure 5B). Inhibition of CAPN10 expression (by doxycycline treatment) decreased insulin-stimulated glucose uptake by approx. 50% compared with untreated adipocytes. The presence of doxycycline had no effect on basal glucose uptake (results not shown). Furthermore, addition of ALLN to doxycycline-treated cells had no further reduction on insulin-stimulated [^{14}C]2-DOG uptake compared with doxycycline-treated cells. To determine whether CAPN10 mediates GLUT4 translocation, 3T3-L1 adipocytes harbouring the transgene were stimulated with insulin, fixed and then probed with an anti-GLUT4 antibody (Figure 5C). Inhibition of CAPN10 expression (by doxycycline treatment) significantly decreased insulin-stimulated GLUT4 translocation to the PM when compared with CAPN10-expressing adipocytes (not treated with doxycycline) (Figure 5C). Inhibition of CAPN10 had no effect on GLUT4 translocation under the basal condition. To determine whether CAPN10 inhibited GLUT4 translocation by disrupting actin reorganization, we stimulated transfected cells with insulin and probed with rhodamine-labelled phalloidin to localize filamentous actin. As shown in Figure 5(D), inhibition of CAPN10 expression (by doxycycline treatment) decreased cortical actin staining when compared with CAPN10-expressing adipocytes (not treated with doxycycline). These studies suggest that CAPN10 plays a specific role in GLUT4 trafficking via actin reorganization.

DISCUSSION

Previous studies have identified CAPN10 as a putative type 2 diabetes susceptibility gene [1]. SNP-43 in CAPN10 was shown to be associated with type 2 diabetes in a population of Mexican–Americans in Starr County, TX, U.S.A. [1]. Further studies have shown a similar association between genetic variations within the CAPN10 gene and the development of type 2 diabetes in other populations [2–5]. Studies in rodents have shown that inhibition of calpain activity increased insulin secretion in isolated rat pancreatic islets and decreased glucose uptake in isolated rat-muscle strips and adipocytes [6]. While the effect of inhibiting calpain activity resulted in the primary characteristics of insulin resistance, it is unclear how calpain functions to alter these parameters at the cellular level.

Our findings show that inhibition of calpain activity in 3T3-L1 adipocytes reduced insulin-stimulated GLUT4 translocation to the PM significantly by disrupting actin filament reorganization (Figures 2, 4 and 5). We also demonstrated that this inhibition of GLUT4 translocation was not mediated by the PI 3-kinase-dependent pathway because inhibition of calpain activity did not prevent the activation of critical signalling proteins in this pathway (Figure 3). These findings suggest that calpain facilitates GLUT4 translocation by disrupting actin reorganization in a PI 3-kinase-independent manner and furthermore, supports the idea that insulin-induced actin reorganization is not mediated by a PI 3-kinase-dependent pathway. Our findings are highly significant in light of the growing number of studies that implicate a role for actin reorganization in insulin-stimulated GLUT4 translocation [18,26,43].

Studies using the actin depolymerizing agent latrunculin B resulted in decreased insulin-stimulated actin reorganization, GLUT4 translocation and glucose uptake [26]. These studies demonstrate that actin polymerization is necessary for GLUT4 translocation. Similarly, our findings show that in the presence of calpain inhibitors, there were decreases in insulin-induced actin filament formation and GLUT4 translocation (Figure 4). Furthermore, specific inhibition of CAPN10 decreased insulin-stimulated GLUT4 translocation and actin reorganization. It is not surprising that inhibition of calpain activity resulted in the disruption of the insulin-induced actin reorganization because calpains have been shown to be involved in actin reorganization during cell migration and focal-adhesion formation [28,37–42]. Studies using calpain-deficient mouse embryonic fibroblasts revealed that these cells were unable to form focal adhesions and further demonstrated the requirement for calpain in the formation of stress fibres [38]. Our results are in agreement with these studies in that inhibition of calpain prevented actin filament formation during insulin-stimulated glucose uptake.

While inhibition of calpain activity blocked GLUT4 translocation, we were unable to detect an increase in calpain activity upon insulin stimulation (Figure 1B). One possible explanation for this finding is that insulin mediates the translocation of an active CAPN10 to its site of action. Previous studies have indicated a relationship between membrane localization of calpain and its activation [44]. Studies are underway to determine the role of insulin on the cellular localization of CAPN10. Alternatively, insulin may cause the translocation of a substrate to calpain or it may mediate a conformational change in a putative substrate making it a target for calpain. Studies are also in progress to investigate these possibilities.

In summary, our studies show a role for calpain activity in insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Furthermore, they demonstrate that calpain facilitates insulin-induced GLUT4 translocation to the PM via actin reorganization, but has no affect on insulin-induced signal transduction along the PI 3-kinase pathway. This study establishes a role for calpain activity in the insulin-induced actin reorganization and GLUT4 vesicle trafficking required for glucose uptake. Determining the mechanism by which calpain activity promotes GLUT4 translocation may provide a cellular basis for impaired insulin sensitivity in individuals with SNP-43. The identification of a calpain-specific target involved in actin reorganization would not only advance our knowledge of the mechanisms underlying insulin resistance, but also provide a possible target for pharmacological intervention.

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