Calpain inhibition impairs glycogen syntheses in HepG2 hepatoma cells without altering insulin signaling

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Abstract

Calpains are a family of non-lysosomal cytoplasmatic cysteine proteases. Since calpain 10 (CAPN10), a member of the calpain family of proteases, has been found to represent a putative diabetes susceptibility gene, it was argued that calpains may be involved in the development of type 2 diabetes. The functional role of calpains in insulin signaling and/or insulin action is, however, not clear. We investigated the effects of the calpains 1 and 2 inhibitor PD151746 on insulin signaling and insulin action in human hepatoma G2 cells (HepG2). HepG2 cells were incubated without (-PD) or with (+PD) 5.33 μmol/l PD151746 for different times and then stimulated with 100 nmol/l insulin for 0 (t0), 5 (t5), 15 (t15), 30 (t30), 45 (t45), and 60 (t60) min. After solubilization of the cells, insulin receptor kinase activity, tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-1-associated phosphatidylinositol-3 kinase (PI3-kinase), PI3-kinase activity, Thr308 phosphorylation of Akt, amount of protein tyrosine phosphatase-ε (PTPε), and glycogen synthase activity were determined. Incubation with PD151746 resulted in a significant reduction of insulin-stimulated glycogen synthesis compared with cells not preincubated with the calpain inhibitor (-PD: t0, 4.90±1.20%; t5, 5.90±1.02%; t15, 5.29±0.95%; t30, 5.60±1.10%; t45, 5.52±0.90%; t60, 5.67±0.97%; +PD: t0, 4.56±1.10%; t5, 6.16±1.05%; t15, 7.52±1.09%; t30, 7.68±1.10%; t45, 8.28±0.89%; t60, 7.69±0.98%; P<0.05). Incubation with PD151746 significantly increased the protein amount of PTPε in the cells after 12 h (-PD: t12, 0.85±0.18 RU (Relative unit); t6, 0.87±0.18 RU; t12, 0.9±0.13 RU; +PD: t12, 0.92±0.21 RU; t6, 1.0±0.15 RU; t12, 1.34±0.16 RU; P<0.05). Calpain inhibition with PD151746 had no effect on the insulin stimulation of the investigated insulin signaling parameters. These results in HepG2 cells suggest that calpains play a role in the hepatic regulation of insulin-stimulated glycogen synthesis independent of the PI3-kinase/Akt signaling pathway.

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Introduction

Type 2 diabetes mellitus is characterized by peripheral insulin resistance, i.e. the loss of an appropriate response to insulin at normal levels. Since the development of type 2 diabetes has a strong genetic background, genes encoding proteins with effects on insulin signaling and/or insulin action are of special interest in order to understand the underlying molecular mechanism of the insulin resistance and to develop effective treatments. Recent studies have identified a member of the calpain family of proteases, CAPN10, as a diabetes susceptibility gene (Horikawa et al. 2000, Cox et al. 2004). 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 clear (Baier et al. 2000, Lynn et al. 2002, Malecki et al. 2002).

Calpains are calcium-activated neutral proteases that catalyze the endoproteolytic cleavage of specific substrates and thereby regulate pathways which affect intracellular signaling. Fourteen members of the calpain family have been identified so far, some of which are tissue specific whereas others are ubiquitously expressed (Goll et al. 2003). Several data of recent studies suggest that calpains are involved in the regulation of glucose homoeostasis. Yang et al. (2001) have shown that a specific single-nucleotide polymorphism–43 (SNP–43) within the CAPN10 gene was associated with elevated fasting blood glucose and insulin levels and decreased CAPN10 mRNA levels in the skeletal muscle of non-diabetic Pima Indians. Furthermore, in vitro studies on effects of CAPN1 (μ-calpain) and/or CAPN2 (m-calpain) inhibitors revealed that inhibition of calpain activity results in increased insulin secretion in isolated rat pancreatic islets (Sreenan et al. 2001, Parnaud et al. 2005) and a significantly decreased insulin–stimulated glucose uptake in isolated rat muscle strips and adipocytes (Sreenan et al. 2001) as well as in 3T3-L1 adipocytes (Paul et al. 2003). These data suggest a potential
link between calpain activity and the insulin signaling pathway resulting in insulin action.

Cellular insulin signaling is initiated by binding to its specific cell surface receptor, followed by rapid phosphorylation of tyrosine residues on the receptor β-subunit. This results in phosphorylation of several intracellular substrates including insulin receptor substrates (IRSs) 1–4. Binding of tyrosine-phosphorylated IRSs to the 85 kD regulatory subunit of the lipid kinase phosphatidylinositol-3 kinase (PI3-kinase) then results in an activation of this enzyme. PI3-kinase is necessary for the insulin-mediated stimulation of the serine/threonine kinase Akt which is activated by phosphorylation at the Thr308 and Ser473 residues (Pirola et al. 2004). Upon activation, Akt phosphorylates and inactivates glycogen synthase kinase-3 (GSK-3), resulting in the dephosphorylation and activation of glycogen synthase (GS; Patel et al. 2004).

In order to assess the role of calpain activity in insulin signaling and insulin action in a human cell model, we investigated whether inhibition of CAPN1 and CAPN2 by PD151746 alters insulin-stimulated glycogen synthesis and/or insulin signal transduction in hepatoma G2 (HepG2) cells.

Materials and Methods

Fetal calf serum (FCS) was purchased from Biochrom (Berlin, Germany), Leibovitz-L15 medium from Gibco. Porcine monocomponent insulin was from Novo Biolabs (Bagsvaerd, Denmark), [125I-Tyr-A14]-monoiodoinsulin from Amersham, [32P]-ATP, and [14C]-glycogen uridine diphosphate glucose (UDPG) were from NEN Life Science Products (Boston, NE, USA). Goat anti-mouse IgG was obtained from Dianova (Hamburg, Germany) and peroxidase-labeled antibodies from Dako (Glostrup, Denmark). Rabbit polyclonal antibodies against IRS-1, PI3-kinase 85 000 Mø regulatory subunit and recombinant IRS-1 were from Upstate Biotechnology (Lake Placid, NY, USA). Antibody against Thr308 phosphorylation of Akt was obtained from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibody against the cytoplasmatic domain of protein tyrosine phosphatase-ε (PTPε) was from Abcam, Inc. (Cambridge, MA, USA). The calpain inhibitor PD151746 as well as m-calpain were obtained from Calbiochem (San Diego, CA, USA). Other chemicals were from Sigma or Roche.

Preparation of PD151746 stock solution and cell incubation

We used HepG2 cells, since these cells represent an established human cell model for the study of cellular insulin signaling and insulin effects. Furthermore, they allow an investigation of long-term effects (Hofmann et al. 1980, Qiu et al. 2004). To prepare the PD151746 stock solution, 1.0 mg PD151746 powder was dissolved in 1 ml dimethylsulfoxide (DMSO)/methanol. This was added to Leibovitz-15 medium and the pH adjusted to 7.4 with NaHCO₃ (final volume 5 ml). Stock solution for controls was similarly prepared except that the DMSO/methanol did not contain PD151746. HepG2 cells that had grown to near confluency (Leibovitz-15 medium supplemented with 10% FCS, 5 mmol/l glucose, 2.5 µl/ml transferrin, 60 µg/ml fetuin, and 20 µg/ml gentamicin at 37 °C under an atmosphere of 95% air and 5% CO₂) were starved for 24 h in Leibovitz-15 medium that contained the same supplements as described above but only 1% FCS (Hofmann et al. 1980). Then, the medium was replaced by 19.9 ml incubation buffer (20 mmol/l HEPES (hydroxyethylpiperazine-ethanesulfonic acid), 130 mmol/l NaCl, 4.8 mmol/l KCl, 1.3 mmol/l KH₂PO₄, 1 g/l d-glucose, 1.3 mmol/l MgSO₄, 1.2 mmol/l CaCl₂, 2% BSA (pH 7.4)) to which 100 µl stock solution without or with PD151746 (final concentration 5.33 µmol/l) had been added and cells were incubated for 1 h. We chose 1 h of incubation without or with 5.33 µmol/l PD151746 because preceding experiments had shown that PD151746 had similar effects on IRS-1 phosphorylation, IRS-1 amount, Thr308 phosphorylation of Akt or on Akt amount after 5 min, 20 min, 4 h, or 12 h and that 0.053, 0.53, or 53.5 µmol/l PD151746 were similarly potent (data not shown). To confirm adequate inhibitor function, casein zymography electrophoresis was performed as described (Raser et al. 1995; Fig. 1).

Cell number and viability

At the end of the incubations, cells were counted in a Neubauer chamber and viability was tested with Trypan blue exclusion.

Insulin stimulation and solubilization of cells

Cells were preincubated as described earlier. Then, incubation buffer with 0 or 100 nmol/l insulin was added and cells incubated for different times at 37 °C. Subsequently, the buffer was removed and the dishes with the cells rapidly frozen in liquid nitrogen. The thin ice layer that contained the cells was scraped off from the dishes at −20 °C and homogenized.

![Calpain inhibition impairs glycogen syntheses](https://www.endocrinology-journals.org)

**Figure 1** In vitro effects of PD151746 in casein zymographies. To confirm adequate inhibitor function, various amounts of calpain (0.1–10 µg, specific activity >1000 units/mg protein) were incubated with or without DMSO (1:100) and with or without the calpain inhibitor PD151746 (5–33 µmol/l) for 5 min. Samples were then electrophoresed into a casein gel (0.2%) and subsequently incubated in a proteolysis buffer for 60 min. The gel was then incubated overnight at room temperature and finally stained with Coomassie blue. Shown is a casein zymogram representative of two separate experiments.
with a motor-driven Potter homogenizer in a solution that contained 2% Triton X-100, 5 mmol/l phenylmethylsulfonyl fluoride, 800 U/ml trypsin inhibitor aprotinin, 8 mmol/l EDTA, 30 mmol/l benzamidine, 2-5 μg/ml pepstatin, 2-5 μg/ml leupeptin, 160 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 0-2 mmol/l sodium vanadate, 2 mmol/l dichloroacetic acid, and 20 mmol/l HEPES (pH 7-4; final concentrations, Schüt et al. 2000). Samples were then centrifuged at $10^5 \text{g}$ (4 °C) to remove insoluble material. Protein concentration was measured using the BioRad protein dye-binding assay.

**Insulin receptor kinase and binding capacities**

These were measured as previously described (Klein et al. 1993). Briefly, 30 μl cell sample lysates were added to microtiter wells coated with anti-insulin receptor antibody for 16 h at 4°C. The wells were washed and receptor-mediated $^{32}$P incorporation into recombinant IRS-1 (17 mmol/l) measured at 120 mmol/l $^{32}$P-ATP $^{[125]}$-Tyr-A$^{141}$-monoiodoinsulin binding to immobilized insulin receptors was also measured in the wells (Klein et al. 1993). Insulin-binding activity (BA) was defined as the amount of specifically bound insulin at a concentration of 8-7 mmol/l and analyzed as described earlier (Klein et al. 1995). Finally, IR kinase activity was expressed as attomoles phosphate incorporated into IRS-1 per minute per femtomole of insulin binding.

**Immunoblots**

Solubilized cells (300 μg protein) were incubated overnight with 1 μg anti-IRS-1 antibody and then added to 30 μl packed agarose beads cross-linked with protein G. Bound proteins were detached with Laemmli buffer, boiled, and subjected to SDS-PAGE. For the determination of Thr$^{308}$-phosphorylated Akt and PTPs, cell lysates (100 μg protein) were directly subjected to SDS-PAGE (7-5%). Proteins were then transferred to nitrocellulose membranes. Membranes were blocked in BSA (3% in Tris (hydroxymethyl) aminomethane buffered saline (TBS–T)) and incubated with the specific antibodies for 2 h. Bound antibodies were detected by peroxidase-labeled antibodies and chemiluminescence.

**IRS-1-associated PI3-kinase activity**

PI3-kinase activity was determined as described (Kolter et al. 1997). Briefly, 60 μl reaction mixture containing 0-2 mg/ml PI, 20 mM HEPES (pH 7-2), 0-4 mM EGTA, 0-4 mM Na$_2$HPO$_4$, and 10 mM MgCl$_2$ with or without wortmannin (1 μM) were added to the immunoprecipitates. The kinase buffer was incubated with the immunoprecipitates for 5 min at room temperature, and the reaction was started by the addition of $[^{32}]$P]ATP (40 μM and 0-2 Ci/μl). After 20 min, the reaction was stopped by the addition of 40 μl of 4 N HCl and 190 μl chloroform–methanol (1/1). The organic phase was extracted and spotted on a silica gel thin-layer chromatography plate (Merck) and was developed in chloroform–methanol–15% NH$_4$OH–water (43:28:5:7, v/v). Plates were dried and radioactivity incorporated into PI quantified by a phospomager.

**Glycogen synthesis**

HepG2 cells were grown and stimulated without or with 100 nmol/l insulin as described earlier. Subsequently, cells were solubilized and GS activity was determined as previously described (Vaag et al. 1992). Briefly, GS activity was assayed in the presence of a near physiological concentration of glucose-6-phosphate (G6P, 0-1 mmol/l) and in the presence of 10 mmol/l G6P to determine maximal enzyme activity. The substrate concentration of UDPG was 7 mmol/l and GS activity was expressed as nmol of UDPG incorporated into glycogen per minute. Fractional velocities (FVs) were calculated as the ratio between GS activities assayed at 0-1 and 10 mmol/l (FV 0-1).

**Data analysis**

In every western blot, all bands were compared (three to four time points $-/+ $ PD151746) by scanning densitometry. Relative units represent the ratio between the intensity of the single bands and the mean intensity of all bands in the same blot. Differences were tested by $t$-statistics for paired data (SigmaStat 3.0, SPSS, Inc., Chicago, IL, USA). $P \leq 0.05$ was considered significant.

**Results**

**Cell number and viability**

PD151746 had no effect on cell number ($1.22 \times 10^5 \pm 0.19$ and $1.25 \times 10^5 \pm 0.13$ cells/dish treated with or without PD151746 respectively) or cell viability ($8.5 \pm 0.16$ and $9.1 \pm 0.12%$ Trypan blue-positive cells/dish respectively). Moreover, there were no detectable morphological changes between cells treated with or without PD151746.

**In vitro calpain activity**

Incubation of 1 and 10 μg calpain with PD151746 for 5 min resulted in a significant irreversible reduction of calpain activity in casein zymographies of at least 80% confirming adequate inhibitor function (Fig. 1).

**Insulin receptor kinase activity, IRS-1 phosphorylation, association of PI3-kinase with IRS-1, PI3-kinase activity, and Thr$^{308}$ phosphorylation of Akt**

In cells not preincubated with PD151746 insulin rapidly increased IRS-1 phosphorylation, association of PI3-kinase with IRS-1, IRS-1–associated PI3-kinase activity, and Thr$^{308}$ phosphorylation of Akt (Figs 2–4). In cells preincubated with...
the calpain inhibitor PD151746, similar activation characteristics of the signal parameters were noticed and no statistical differences were detected between cells treated with or without the inhibitor (Figs 2–4). PD151746 had no effects on the amounts of IRS-1 or Akt (data not shown).

**Glycogen synthesis**

Insulin stimulation increased GS activity in cells not preincubated with PD151746 by 25–45%. The greatest effects were observed after 45 min. Preincubation with PD151746 resulted in a significant reduction of insulin-stimulated GS activity after 15, 30, 45, and 60 min by 30–40% respectively (Fig. 5).

**Protein expression of PTPε**

In cells preincubated for 1 h with the calpain inhibitor PD151746, insulin stimulation did not alter the amount of the PTPε protein (Fig. 6A). However, the incubation of HepG2 cells with PD151746 for 8 or 12 h resulted in an increase of the PTPε protein expression by 20–30% respectively. This alteration in the amount of the PTPε protein was significant after an incubation period of 12 h (P=0.035; Fig. 6B).

**Discussion**

In this study, we describe that the incubation of HepG2 cells with the calpain inhibitor PD151746 over a period of...
15–60 min resulted in an approximately 40% reduction of insulin-stimulated GS activity respectively. A similar result with a highly reduced insulin-stimulated glycogen synthesis rate after CAPN2 inhibition was also found in a recent study by Sreenan et al. (2001) in rat muscle strips.

The effect of calpain inhibition with PD151746 on insulin-stimulated GS activity in HepG2 cells was not associated with alterations in the insulin stimulation of the insulin receptor kinase activity, tyrosine phosphorylation of IRS-1, IRS-1-associated PI3-kinase, PI3-kinase activity, or Thr308 phosphorylation of Akt. These data suggest that the PD151746 effect was caused by alterations at non-studied locations, e.g., downstream signaling elements (Pirola et al. 2004) or the final effector system itself (Patel et al. 2004). A similar result with a decreased insulin effect on glucose homeostasis independent of the PI3/Akt-kinase pathway was found in a clamp study on insulin-resistant subjects with type 2 diabetes and first-degree relatives of type 2 diabetic patients. Although the glucose disposal was reduced in the diabetic subjects and relatives, no alterations in the insulin-stimulated PI3-kinase activation and Thr308 Akt phosphorylation were found in muscle biopsies obtained at similar steady-state clamp insulin concentrations (Meyer et al. 2002). Another recent study investigated the effect of the insulin signaling-independent muscle contraction-stimulated glucose transport in transgenic mice that overexpress the calpain inhibitor calpastatin. Despite a three- to fourfold increase in glucose transporter-4 protein, calcium calmodulin kinase II and AMP kinase in their skeletal muscles, contraction-stimulated glucose transporter-4 translocation, and glucose transport were not increased above wild type values (Otani et al. 2006). These findings also suggest that the inhibition of calpain results in alterations of a step downstream of the insulin signaling pathways and/or at the level of the final effector system itself.

A potential candidate for such an effect is the PTPε that is known to affect the hepatic glycogen synthesis. Recent studies in primary rat hepatocytes revealed that the expression of PTPε inhibited insulin signaling and GSK-3β mediated hepatic glycogen synthesis (Nakagawa et al. 2005). Moreover, cytosolic PTPε does not inhibit insulin receptor signaling but dephosphorylates several other signaling molecules (Andersen et al. 2001). Since PTPε is also cleaved by calpains (Gil-Henn et al. 2001), inhibition of calpain activity might result in an altered PTPε protein amount and/or an increased phosphatase activity, and, thus, might reduce GS activity without altering insulin signaling. Our result, that the PTPε protein amount was not altered by the calpain inhibitor after 30 and 60 min, does not support a role of PTPε as a mechanism of a

**Figure 3** Effects of PD151746 on IRS-1 phosphorylation (A), association of p85 with IRS-1 (B) and Thr308 phosphorylation of Akt (C). Cells were incubated without (○) or with (●) 5·33 μmol/l PD151746 for 1 h and then stimulated with 100 nmol/l insulin for the indicated times. They were then solubilized and IRS-1 immunomobilized with anti-IRS-1 antibody bound to protein G agarose beads. The beads were washed, bound proteins detached with Laemmli buffer, and immunobLOTS performed as described in Materials and Methods. Thr308-phosphorylated Akt in cell lysates was measured by immunoblot with specific anti-phospho-Akt antibody. Relative units (RUs) were calculated as described. Shown are means ± S.E.M. (n=5). Insets show representative blots at the respective time points.

**Figure 4** Effects of PD151746 on PI3-kinase activity. Cells were incubated without (○) or with (●) 5·33 μmol/l PD151746 for 1 h and then stimulated with 100 nmol/l insulin for the indicated times. They were then solubilized and IRS-1 was immunomobilized as described in Materials and Methods. Subsequently, 60 μl reaction buffer containing PI were added to the immunoprecipitates and the reaction was started by the addition of [γ-32P]ATP. After 20 min, reaction was stopped by the addition of 40 μl HCl (4 mol/l) and 190 μl chloroform–methanol. The organic phase was extracted and spotted on a chromatography plate. Plates were developed in chloroform–methanol and radioactivity incorporated into phosphatidylinositol quantified by phosphoimaging. Shown are means ± S.E.M. (n=6).

**Figure 5** Effects of PD151746 on glycogen synthesis. HepG2 cells were incubated without (○) or with (●) 5·33 μmol/l PD151746 for 1 h and then stimulated with 100 nmol/l insulin for the indicated times. Cells were solubilized and reaction was started by adding a reaction mixture containing 25 mM NaF, 20 mM EDTA, 1% glycerogen, 0·7 μCi U-[14C]UDP glucose, 0·3 mM UDP glucose, and 0·1 mM G6P was added. Reaction was stopped after 15 min by precipitation of the total glycogen. Glycogen synthease activity was assayed in the presence of a near physiological concentration of G6P (0·1 mM) and in the presence of 10 mmol/l G6P to determine maximal enzyme activity.
reduced GS activity within this time period. An incubation period of 8 or 12 h, however, increased the PTP3 protein amount. These data confirm the result of a study in human embryonic kidney HEK 293 cells suggesting that PTP3 is cleaved by calpain (Gil-Henn et al. 2001). They also demonstrate that the calpain inhibitor PD151746 was effective in the HepG2 cells at all. Such a long-lasting calpain inhibitor effect on the PTP3 protein amount may induce or enhance alterations of the GS activity and/or insulin signaling. However, since the PTP3 protein amount was not altered after a calpain incubation period that clearly reduced the GS activity in HepG2 cells, the PTP3 appears not to be involved. In any case, these data do not exclude a potential role of other phosphatases or a direct inhibition of the PTP3 activity.

Another potential mechanism of a PI3-kinase-independent impairment of insulin-stimulated glycogen synthesis is the insulin-dependent pathway that mediates cytosolic actin reorganization. Actin filaments are an essential part of the cytoskeleton and are regulated by ubiquitous calpain isoforms (Mazeres et al. 2006). Actin filament formation facilitates insulin-stimulated GLUT4 translocation (Bose et al. 2002) and modulates the initiation of insulin-stimulated glycogen synthesis (Baque et al. 1997, Prats et al. 2005). Thus, disturbances of actin reorganization by inhibition of calpains might alter these insulin effects. This hypothesis is supported by a study by Paul et al. (2003), who observed that calpain inhibition reduced insulin-stimulated glucose uptake in 3T3 adipocytes. The authors showed that a reduction of insulin-stimulated glucose uptake by calpain inhibition was not related with alterations of the insulin-stimulated PI3-kinase pathway but with a calpain–associated disruption of the insulin-mediated actin reorganization (Paul et al. 2003). Since actin cytoskeleton remodeling plays an important role in insulin-stimulated hepatic glycogen metabolism as well (Huang et al. 2002, Prats et al. 2005), calpain inhibition might, by this, also result in a PI3/Akt-kinase-independent impairment of glycogen synthesis in HepG2 cells.

It is also possible that calpains affect target proteins downstream of Akt. One such potential target proteins is the casein kinase 2 (CK2) that rapidly phosphorylates GS. This ‘priming’ phosphorylation is necessary to initiate further phosphorylation of GS by GSK-3 (Patel et al. 2004). Since CK2 is cleaved by CAPN2 (Roig et al. 1999), inhibition of calpains might increase GS ‘priming’ phosphorylation by CK2 and, consequently, enhance GS phosphorylation by GSK-3 resulting in inhibition of GS activity.

Finally, our data suggest that CAPN1 and CAPN2 are involved in the regulation of insulin-stimulated glycogen synthesis in human liver cells independently of the PI3-kinase/Akt signaling pathway. Such an inhibition of calpain activity resulting in impaired hepatic glycogen synthesis might represent a potential mechanism that could contribute to the hepatic insulin resistance in type 2 diabetes.

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