

Sequential cleavage of insulin receptor by calpain 2 and γ -secretase impairs insulin signalling

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Received: 8 July 2016 / Accepted: 8 August 2016 / Published online: 30 September 2016
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Abstract

Aims/hypothesis Soluble insulin receptor (sIR), the ectodomain of the insulin receptor (IR), has been detected in human plasma and its concentration paralleled that of blood glucose. We have previously developed an in vitro model using HepG2 liver-derived cells, which mimics changes in sIR levels in plasma from diabetic patients and shows that calcium-dependent proteases cleave IR extracellularly (a process known as shedding). The present study aimed to reveal the mechanisms of IR cleavage.

Methods Using the in vitro model, we investigated the molecular mechanisms of IR cleavage, which is accelerated by high-glucose treatment. We also analysed the relationship between

IR cleavage and cellular insulin resistance, and the correlation between plasma sIR levels and insulin sensitivity, which was assessed by the euglycaemic–hyperinsulinaemic clamp technique.

Results Here, we determined that calpain 2, which is secreted into the extracellular space associated with exosomes, directly cleaved the ectodomain of the IR β subunit (IR β), which in turn promoted intramembrane cleavage of IR β by γ -secretase. IR cleavage impaired insulin signalling and the inhibition of IR cleavage (by knockdown of calpain 2 and γ -secretase), restored IR substrate-1 and Akt, independent of IR. Furthermore, the glucose-lowering drug, metformin, prevented IR cleavage accompanied by inhibition of calpain

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Electronic supplementary material The online version of this article (doi:10.1007/s00125-016-4102-5) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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2 release in exosomes, and re-established insulin signalling. In patients with type 2 diabetes, plasma sIR levels inversely correlated with insulin sensitivity.

Conclusions/interpretation Sequential cleavage of IR by calpain 2 and γ -secretase may contribute to insulin signalling in cells and its inhibition may be partly responsible for the glucose-lowering effects of metformin. Thus, IR cleavage may offer a new mechanism for the aetiology of insulin resistance.

Keywords Calpain 2 · Exosome · Insulin resistance · Metformin · Shedding · Soluble insulin receptor · Type 2 diabetes

Abbreviations

AMPK	5' AMP-activated protein kinase
BAPTA-AM	<i>O,O'</i> -Bis [2-aminophenyl] ethyleneglycol- <i>N,N,N',N'</i> -tetraacetic acid tetraacetoxymethyl ester
DON	Deoxynorleucine
ERK	Extracellular signal-regulated kinase
ICD-IR β	Intracellular C-terminal domain of the β subunit of the insulin receptor
IR	Insulin receptor
IR β	IR β subunit
<i>O</i> -GlcNAc	<i>O</i> -linked <i>N</i> -acetylglucosamine
sIR	Soluble insulin receptor
siRNA	Small interfering RNA
TSAP6	Tumour suppressor-activated pathway 6

Introduction

Insulin signalling exerts pleiotropic effects on cellular metabolism, growth and differentiation. Impaired insulin signalling causes insulin resistance [1, 2] and several mechanisms have been postulated to explain its pathogenesis [3]. Nevertheless, the causative molecule of human insulin resistance has not been identified [4].

Insulin binds to the insulin receptor (IR) on the plasma membrane and activates tyrosine kinase, which consequently initiates the intracellular signalling pathway [5]. We found that soluble IR (sIR), the ectodomain of IR, exists in human plasma and its concentration paralleled that of blood glucose [6]. We also found that human primary hepatocytes and endothelial cells generated sIR and from this we developed an *in vitro* model that mimicked changes in sIR levels in plasma from patients with diabetes [7]. This model demonstrated that IR underwent proteolytic cleavage extracellularly, on the plasma membrane. However, it remains to be determined how IR is cleaved.

The soluble ectodomains of several membrane receptors have been detected in plasma samples. Most of these

ectodomains are released by proteolytic cleavage of the membrane receptor, a process also known as shedding. The physiological implications of receptor cleavage are diverse. In some cases, the soluble receptor may function as a decoy receptor, which inhibits the activities mediated by binding of their ligands [8, 9]. Membrane receptor cleavage can also downregulate ligand signalling by decreasing the amount of intact receptor localised on the cell membrane [10]. In other cases, ectodomain shedding is followed by intramembrane proteolysis and translocation of the intracellular domain to the nucleus, where it acts as a transcription factor [11, 12]. However, the physiological role underlying IR cleavage has not been defined.

Here, we present the molecular mechanisms of sIR generation and the biological role of IR cleavage in impaired insulin receptor signalling in cells cultured in high-glucose conditions. We also analysed the functional role of biguanides in IR cleavage and investigated the correlation between plasma sIR levels and insulin sensitivity in patients with type 2 diabetes.

Methods

Materials The following antibodies and reagents were purchased from Cell Signaling Technology (Beverly, MA, USA): (1) rabbit monoclonal antibodies against presenilin 1 (D39D1), His-tag (D3I10), p-IRS-1 (Ser612; C15H5), p-Akt (Ser473; D9E), p-Akt (Thr308; C31E5E), Akt (C67E7), p-p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase [ERK] 1/2; Thr202/Tyr204; D13.14.4E) and p44/42 MAPK (137 F5); (2) rabbit polyclonal antibodies against calpain 2 large subunit (M-type) and p-IRS1 (Ser636/639); and (3) mouse monoclonal antibodies against the IR β subunit (IR β ; L55B10) and p-tyrosine (P-Tyr-100). Rabbit polyclonal antibodies against CD63 were purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibodies against IRS1 (06-248), recombinant rat calpain-2 and MG132 were purchased from Merck-Millipore (Billerica, MA, USA). Rabbit polyclonal antibodies against IR β (C-19 and SC-711) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies against β -tubulin (TUB 2.1) and deoxynorleucine (DON), L-685,458 and metformin (1,1-dimethylbiguanide hydrochloride) were purchased from Sigma-Aldrich (St Louis, MO, USA). *O,O'*-Bis [2-aminophenyl] ethyleneglycol-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) were purchased from Dojindo (Kumamoto, Japan). Bufornin hydrochloride was purchased from Wako (Osaka, Japan). Recombinant human EGF was purchased from Pepro Tech (Rocky Hill, NJ, USA). Mouse monoclonal antibodies against the IR α subunit (5D9) were validated [13] and donated by R. A. Roth (Stanford School of Medicine, Stanford, CA, USA). All other antibodies were

validated by their manufacturers. All other reagents used were of analytical grade.

Cell culture HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were grown as previously described [7]. In brief, HepG2 cells were grown in DMEM basal glucose (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FBS (vol./vol.) and were cultured at 37°C in 5% CO₂. MG132 was used to prevent proteasome degradation of the intracellular C-terminal domain of IR β (ICD-IR β). Mycoplasma contamination was not determined.

Immunoprecipitations and western blot analysis Cell lysates were prepared and equal amounts of protein were subjected to immunoprecipitation with specific antibodies as previously described [14]. In brief, cell lysates were prepared with lysis buffer (1% (wt/vol.) Nonidet P-40, 50 mmol/l Tris-HCl, pH 7.4, 140 mmol/l NaCl, 10 mmol/l EDTA, 20 mmol/l sodium fluoride, 1 mmol/l sodium orthovanadate, 20 mmol/l β -glycerophosphate, 1 mmol/l phenylmethylsulfonyl fluoride, 4 μ g/ml aprotinin, 4 μ g/ml leupeptin and 4 μ g/ml Pepstatin A), and the precipitate was removed by centrifugation at 16,100 \times g for 20 min at 4°C. Equal amounts of protein were subjected to immunoprecipitation with specific antibodies and Protein A-Sepharose CL-4B or Protein G-Sepharose 4 Fast Flow (GE Healthcare, Little Chalfont, UK). Extracts from the immunoprecipitates or equal amounts of cell lysates were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and probed with specific antibodies. All primary antibodies were used at a 1:1000 dilution. Proteins were visualised using secondary antibody (horse-radish peroxidase conjugated goat anti-rabbit or mouse IgG, at 1:2000 dilution: Medical & Biological Laboratories, Nagoya, Japan) and enhanced chemiluminescence (ECL Prime; GE Healthcare). A representative immunoblot for each western blot analysis is shown.

Ultrasensitive ELISA for human sIR We used an ultrasensitive ELISA (immune complex transfer enzyme immunoassay) to measure the levels of human sIR, as previously described [15]. In brief, the sIR-antibody immune complexes were transferred from one solid phase to another solid phase. Two kinds of monoclonal antibodies against the human specific IR α subunit were used: 5D9 (gifted from Medical & Biological Laboratories) and 83-7 (purchased from Lab Vision, Fremont, CA, USA). The amount of intact IR in cell lysates was also determined using this assay. The net increase in IR cleavage was calculated by dividing the amount of sIR in the medium with that of IR in cell lysates, relative to that in control cells.

siRNA knockdown Ambion small interfering (si)RNAs for human *CAPN2* (-1, S319; -2, S320; -3, S321) and *PSEN1* (-1, S112; -2, S113) and the negative control (no. 1) siRNA were purchased from Thermo Scientific (Waltham, MA, USA). The

knockdown protocol using these siRNAs has been previously described [7]. In brief, HepG2 cells were transfected with the indicated siRNAs (100 nmol/l). For insulin stimulation, the medium was replaced every 24 h with DMEM containing 5.5 or 16.5 mmol/l glucose plus 1% FBS (vol./vol.), which was followed by incubation in serum-free DMEM for 1 h.

Isolation of exosomes from HepG2 culture medium

Exosomes from the HepG2 culture medium were isolated using total exosome isolation Reagent (from cell culture media) (Thermo Scientific), according to the manufacturer's instructions with minor modifications. In brief, HepG2 cells were seeded in 10 cm dishes and incubated with DMEM containing 5.5 or 16.5 mmol/l glucose and 1% FBS (vol./vol.) with or without 3 mmol/l metformin or 5 μ mol/l DON for 72 h. After washing the cells with DMEM, 10 ml DMEM without serum was added to each dish for an additional 24 h. The cell media were centrifuged at 10,000 g for 30 min to remove cells and debris.

Calpain activity assay To measure the proteolytic activity of calpain 2, recombinant IR containing His-tag at the C-terminal of the IR β ectodomain (Sino Biological, Beijing, People's Republic of China) was used as a substrate. Recombinant calpain 2 (2 μ g) (Merck-Millipore) was incubated with the substrate (1.5 μ g) in 20 mmol/l Tris-HCl (pH 7.4), 1 mmol/l EGTA, with or without 3 mmol/l CaCl₂, at 25°C for 20 min. Reactions were stopped by adding SDS sample buffer. Proteolytic activity was evaluated by western blotting using antibodies against His-tag, in which disappearance of the His-tagged IR β ectodomain indicated its cleavage by calpain 2. Coomassie staining was performed according to a standard protocol [16].

Euglycaemic–hyperinsulinaemic clamp study Sixteen Japanese participants with type 2 diabetes were recruited. The patients with advanced diabetic complications, such as unstable retinopathy and severe kidney dysfunction, and severe liver dysfunction were excluded. Their characteristics are summarised in the electronic supplementary material (ESM) Table 1. A euglycaemic–hyperinsulinaemic clamp study was performed in each patient after >1 week of intensive insulin therapy to achieve target blood glucose levels (pre-meal blood glucose <7 mmol/l and 2 h post-meal blood glucose <10 mmol/l). The target plasma glucose and insulin levels during the euglycaemic–hyperinsulinaemic clamps were 5.5 mmol/l and 600 pmol/l, respectively. When the exogenous glucose infusion rate reached a steady state, we determined insulin sensitivity as the metabolic clearance rate of glucose, which was calculated as the average endogenous glucose infusion rate divided by glucose concentration. We also measured plasma sIR concentrations at the start of the glucose clamp test to assess insulin sensitivity simultaneously.

Pearson's correlation coefficient was used to determine the association between sIR level and metabolic clearance rate of glucose. The study was approved by the Ethical Committee for Human Studies at Tokushima University Hospital. Written informed consent was obtained from each patient after receiving a detailed written explanation of the study.

Statistical analysis No data were excluded and all results presented were substantiated by repeated experiments. Experiments were performed and analysed in a blinded fashion when possible. Values are presented as mean \pm SEM. Data involving more than two groups were analysed with ANOVA significance levels were set at <0.05 , <0.01 and <0.001 .

Results

Calpain 2 cleaves the extracellular domain of IR We have previously reported that the putative membrane protease that is assigned to cleave IR was dependent on calcium ions [7]. As a calcium-dependent protease, calpain performs proteolytic processing rather than degradation, and participates in a variety of biological events. To test the possibility that IR β is a calpain substrate, we analysed it using the calpain database (Calpain for Modulatory Proteolysis Database [CaMPDB]) [17]. The result showed that IR β has some predicted sequences for calpain proteolysis, which are located on the N-terminal side of the transmembrane domain in the β subunit. Among the human calpains, calpain 2 is expressed extracellularly and intracellularly, making it a candidate for IR cleavage. Knockdown of calpain 2 using siRNA significantly decreased the amount of sIR in the medium (Fig. 1a) and inhibited the high-glucose-induced proteolytic effect on IR cleavage (Fig. 1b). In vitro, the calpain activity assay demonstrated that the C-terminal His-tag of the recombinant IR β ectodomain was removed in a calcium ion-dependent manner (Fig. 1c). Moreover, the IR β ectodomain in which the His-tag was deleted could be observed in the Coomassie-stained gel (Fig. 1c). These results demonstrate that calpain 2 directly catalyses IR cleavage at a specific site of the IR β ectodomain.

Sequential cleavage of IR by calpain 2 and γ -secretase A previous report has demonstrated that regulated intramembrane proteolysis by γ -secretase is preceded and regulated by an initial distinct cleavage in a process termed ectodomain shedding [18]. Knockdown of calpain 2 decreased the accumulation of ICD-IR β (Fig. 1d), which has been shown to be digested by γ -secretase [7]. The high-glucose-induced accumulation of ICD-IR β was also decreased by EGTA, but not by the intracellular calcium chelator, BAPTA-AM (Fig. 1e). Collectively, IR proteolysis by calpain 2 occurs in the extracellular space and is sequentially followed by intramembrane cleavage by γ -secretase (schematic shown in Fig. 1f).

Exosome-associated calpain 2 is released into cell media

Our findings demonstrate that IR cleavage performed by calpain 2 occurs extracellularly. Because exosomes have been found to be released into HepG2 cell medium [19], we investigated whether calpain 2 is secreted into the extracellular space associated with exosomes. We found that high-glucose treatment increased the amount of calpain 2 in the HepG2 culture medium; however, there was no significant difference in the amount of exosomes between basal and high-glucose treatment (as indicated by CD63 levels in Fig. 2a).

O-linked *N*-acetylglucosamine (*O*-GlcNAc) modification has been shown to be involved in the glucose dependency of IR cleavage [7]. Chemical inhibition of *O*-GlcNAc modification by DON suppressed the increment of calpain 2 relative to the reduction in exosomes, suggesting that calpain 2 release is modified by *O*-GlcNAcylation (Fig. 2b). Additionally, high-glucose treatment significantly decreased intracellular calpain 2 expression, which was recovered by DON treatment (Fig. 2b). These results indicated that *O*-GlcNAc modification facilitated exosome-related calpain 2 release, accompanied by a decrease in intracellular calpain 2 expression, and that its inhibition restored the amount of calpain 2 in the cells.

IR cleavage impairs insulin receptor signalling To elucidate how cellular physiology was impacted by IR cleavage, the relationship between IR cleavage and insulin signalling in cells was analysed. Glucose at ≥ 11 mmol/l significantly induced IR cleavage (Fig. 3a), and at higher concentrations (≥ 16.5 mmol/l) glucose decreased the phosphorylation levels of Akt and ERK, two representative components of the insulin signalling pathway (Fig. 3b).

A previous study has reported that high glucose suppresses insulin signalling in HepG2 cells [20]. It has been subsequently reported that Akt1 and Akt2 are modified by *O*-GlcNAc, which attenuates Akt phosphorylation [21]. Akt is a component of various growth factor signalling pathways. Phosphorylation of Akt by EGF was not inhibited by a high glucose concentration (Fig. 3c), indicating that, in our model, direct *O*-GlcNAc modification is not responsible for the decreased phosphorylation of Akt, and that IR cleavage may cause selective dampening of insulin signalling.

EGTA extracellularly inhibits the calcium-dependent protease that cleaves IR [7]. EGTA partially rescued the high-glucose-induced suppression of insulin-induced Akt phosphorylation (Fig. 3d). Furthermore, knockdown of calpain 2 restored Akt phosphorylation (Fig. 3e). Moreover, knockdown of presenilin 1, which contains the active site of γ -secretase [22], enhanced Akt phosphorylation (Fig. 3f). These results demonstrate that sequential cleavage of IR is involved in the impaired insulin signalling induced by high glucose in cells.

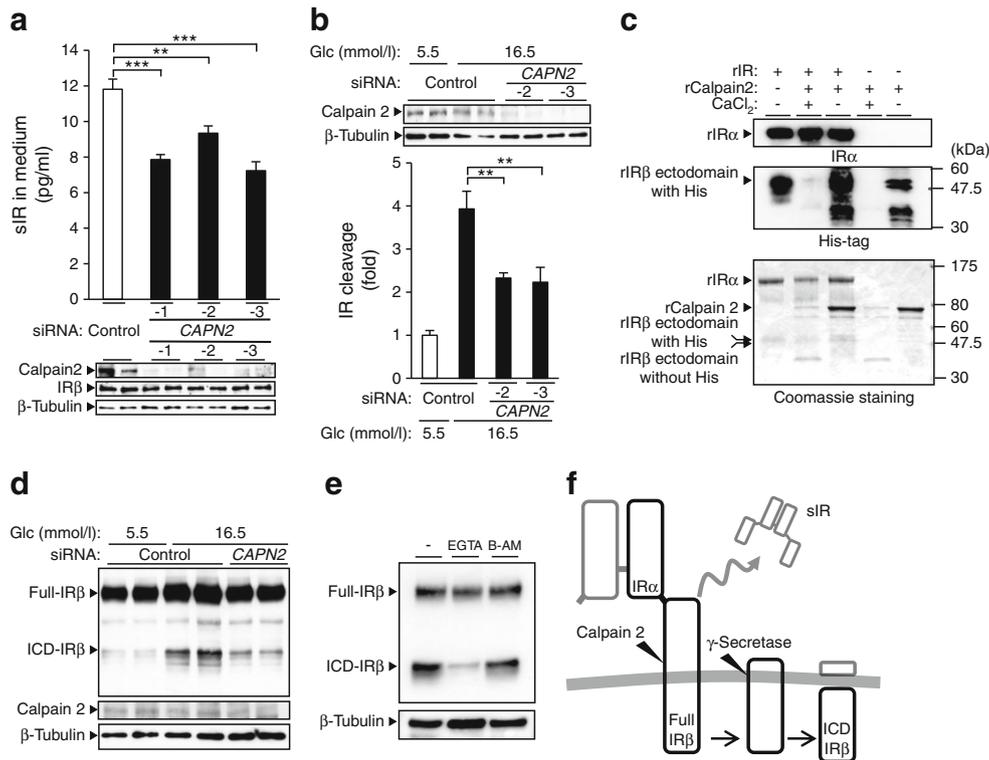
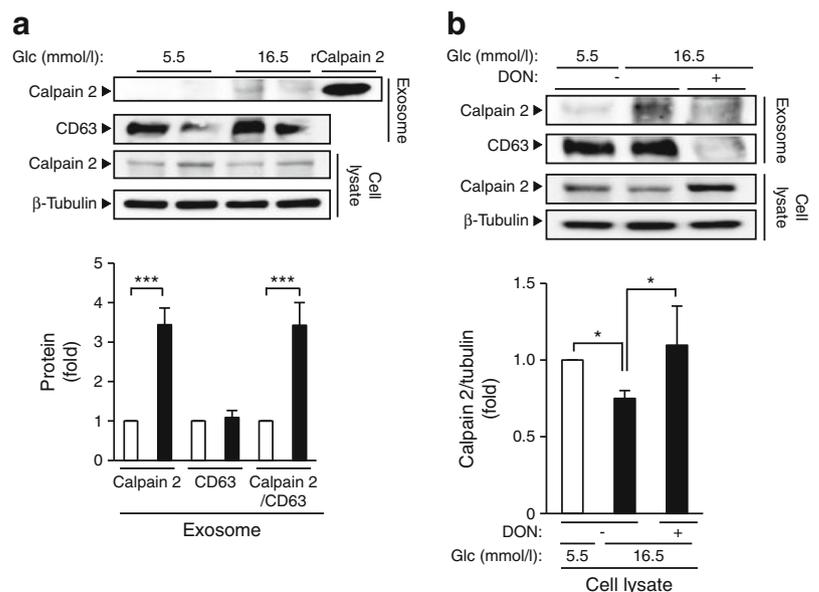


Fig. 1 Sequential cleavage of IR by calpain 2 and γ -secretase. **(a)** Role of calpain 2 on sIR generation. Following knockdown of calpain 2, cells were treated with 5.5 mmol/l glucose for 48 h. Subsequently, cells were incubated for an additional 24 h and media and cell lysates were prepared ($n=6$). **(b)** IR cleavage relative to control (5.5 mmol/l glucose). Following knockdown of calpain 2, cells were treated with 5.5 or 16.5 mmol/l glucose for 48 h. Subsequently, cells were incubated for an additional 24 h and media and cell lysates were prepared ($n=6$). **(c)** In vitro calpain activity, as demonstrated by immunoblotting of IR α and His-tag ($n=3$). **(d)** Sequential cleavage of IR. Following knockdown

of calpain 2 and incubation with glucose for 48 h, cells were incubated in serum-free DMEM containing 10 μ mol/l MG132 for an additional 24 h ($n=3$). **(e)** Extracellular cleavage of IR. Following incubation with 16.5 mmol/l glucose for 72 h, cells were incubated in serum-free DMEM containing 5 μ mol/l MG132 with 5 mmol/l EGTA or 5 μ mol/l BAPTA-AM for an additional 24 h ($n=5$). **(f)** Schematic representation of the proposed sequential cleavage model of IR by calpain 2 and γ -secretase. B-AM, BAPTA-AM; Glc, glucose; rCalpain 2, recombinant calpain 2; rIR, recombinant IR. $**p < 0.01$, $***p < 0.001$

Fig. 2 Calpain 2 release with exosomes. Following incubation with glucose (Glc) for 96 h, cells were incubated for an additional 24 h in **(a)** serum-free DMEM ($n=6$) or **(b)** serum-free DMEM with or without 5 μ mol/l DON ($n=3-7$), prior to preparation of exosomes from media and cell lysates. White bars, 5.5 mmol/l glucose; black bars, 16.5 mmol/l glucose. $*p < 0.05$, $***p < 0.001$



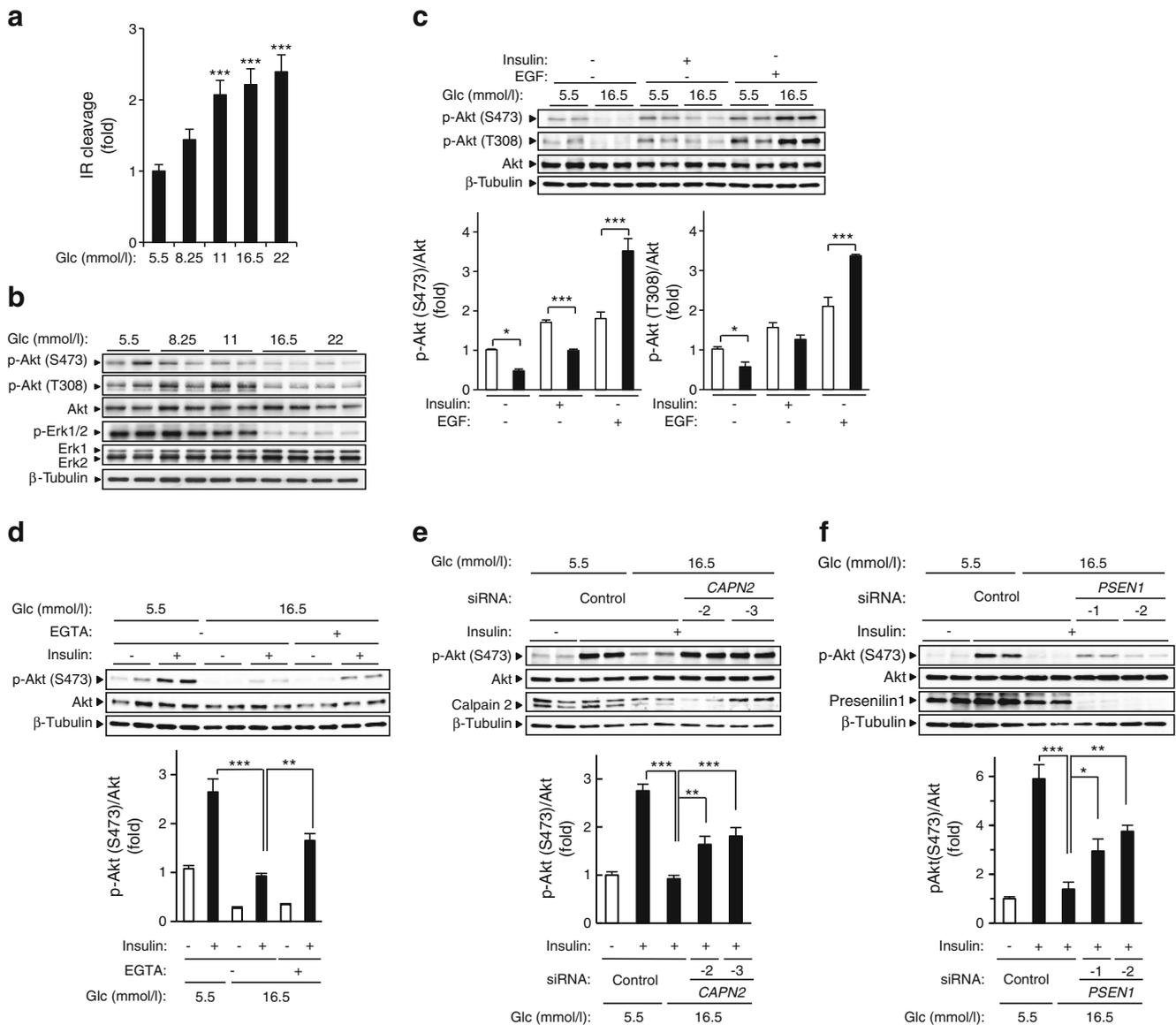


Fig. 3 IR cleavage impairs the insulin receptor signalling pathway. **(a)** IR cleavage relative to control (5.5 mmol/l glucose). Following incubation with glucose for 72 h, cells were incubated for an additional 24 h, prior to media preparation ($n=11$). **(b)** Impaired insulin receptor signalling. Following incubation with glucose for 72 h, cells were incubated for an additional 24 h, prior to cell lysate preparation. **(c, d)** Specific inhibition of insulin receptor signalling. Following incubation with glucose for 72 h, cells were incubated in **(c)** serum-free DMEM, or **(d)** serum-free DMEM

with or without 5 mmol/l EGTA for 5 h, and subsequently stimulated with **(c, d)** 10^{-9} mol/l insulin or **(c)** 500 ng/ml EGF for 30 min. **(c)** $n=4-6$; **(d)** $n=4$. **(e, f)** Enhanced insulin sensitivity by inhibition of IR cleavage. Following knockdown with siRNA and incubation with glucose for 72 h, cells were stimulated with 10^{-9} mol/l insulin for 30 min ($n=3-5$). White bars, 5.5 mmol/l glucose; black bars, 16.5 mmol/l glucose. Glc, glucose. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Metformin suppresses IR cleavage and ameliorates high-glucose-induced impairment of insulin signalling Biguanides, including buformin and especially metformin, are known to improve insulin sensitivity and are widely used as the first-line drug for type 2 diabetes [23]. Based on the hypothesis that these drugs inhibit IR cleavage, we evaluated them in our model. Both drugs significantly suppressed the high-glucose-induced increase in IR cleavage in HepG2 cells (Fig. 4a, b). Metformin suppressed the exosome-associated calpain 2 release induced by high-glucose treatment (Fig. 4c). Concomitantly,

metformin restored phosphorylation levels of IRS1, Akt and ERK, which were suppressed by high glucose (Fig. 4d, g), and enhanced insulin-mediated Akt phosphorylation (Fig. 4e).

To determine whether the IR signal is responsible for the cellular insulin resistance induced by IR cleavage, the cell surface's IR content and tyrosine phosphorylation of IR β following sIR generation were investigated (Fig. 4f). The cell surface expression and autophosphorylation of IR β were not significantly decreased following cleavage, indicating that impaired insulin signalling induced by IR cleavage is not a result of IR signalling

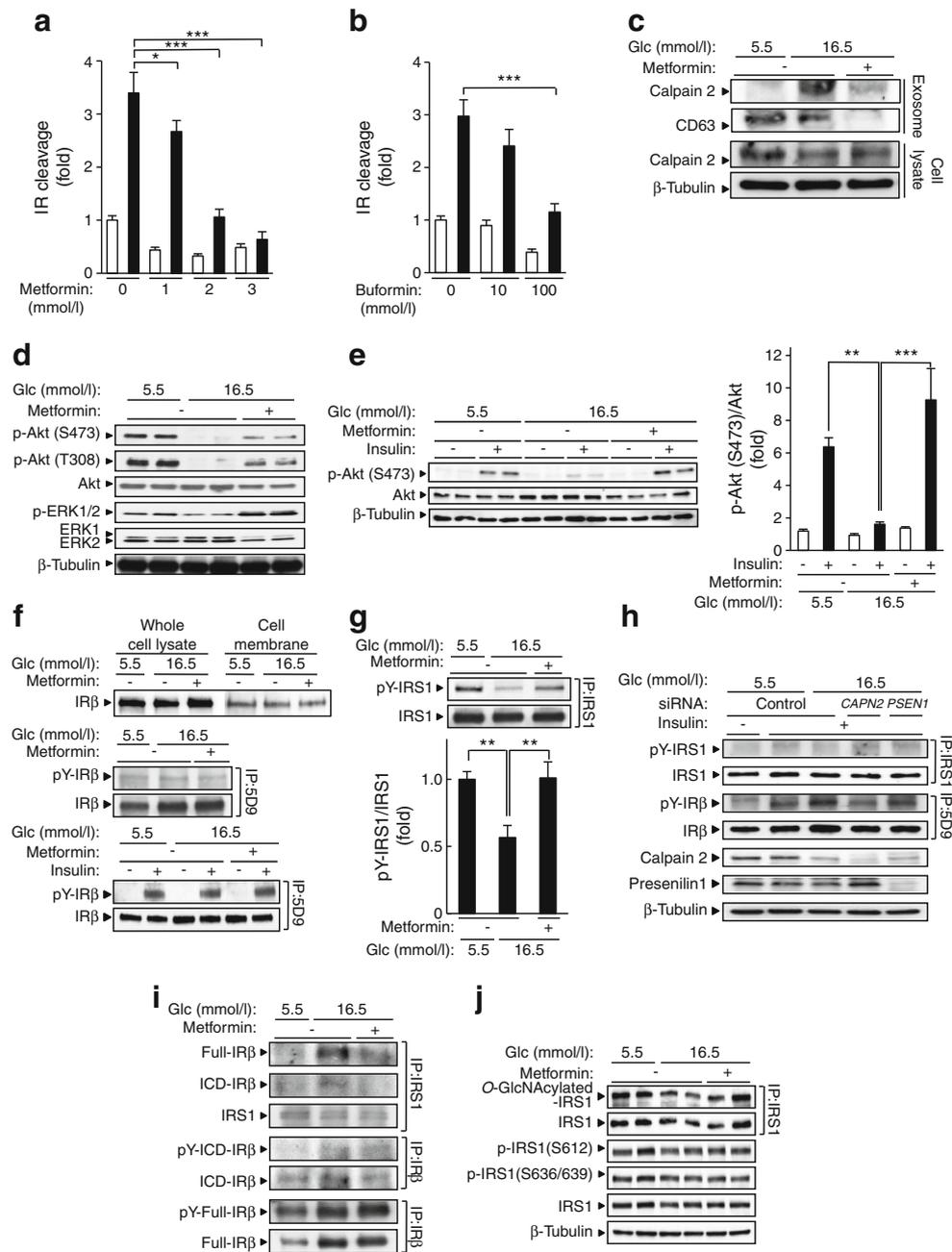


Fig. 4 Pharmacological inhibition of IR cleavage using metformin ameliorates high-glucose-induced impairment of insulin signalling. **(a, b)** IR cleavage relative to control (control conditions: 5.5 mmol/l glucose without **(a)** metformin or **(b)** buformin). Following incubation with glucose and the indicated concentrations of metformin or buformin for 72 h, cells were incubated for an additional 24 h prior to media and cell lysate preparation ($n = 8-9$). **(c)** Inhibition of exosome release by metformin. Following incubation with glucose with or without 3 mmol/l metformin for 96 h, cells were incubated in serum-free DMEM for an additional 24 h and then exosomes from media and cell lysates were prepared. **(d, e)** Enhanced insulin receptor signalling by metformin. **(d, e)** Cells ($n = 4$) were incubated with glucose with or without 3 mmol/l metformin for 96 h, **(e)** followed by incubation in serum-free DMEM for 1 h and subsequently stimulation with 10^{-9} mol/l insulin for 30 min. **(f)** IR signalling on the cell surface. Cells were incubated with glucose with or without 3 mmol/l metformin for 96 h, followed by biotinylation of cell surface

proteins, preparation of cell lysates, or stimulation with 10^{-8} mol/l insulin for 30 min ($n = 2-3$). **(g)** Enhanced IRS1 signalling by metformin. Cells were incubated with glucose with or without 3 mmol/l metformin for 96 h ($n = 7-8$). **(h)** Enhanced IRS1 signalling by inhibition of IR cleavage. Following knockdown with siRNA and incubation with glucose for 96 h, cells were stimulated with insulin at 10^{-9} mol/l (for pY-IRS1 analysis) or 10^{-8} mol/l (for pY-IRβ analysis) for 30 min. ($n = 3$) **(i)** ICD-IRβ binding to IRS1. Following incubation with glucose with or without 3 mmol/l metformin for 96 h, cells were incubated with 5 μmol/l MG132 for an additional 24 h ($n = 3$). **(j)** O-GlcNAcylation and serine phosphorylation of IRS1. Cells were incubated with glucose with or without 3 mmol/l metformin for 96 h ($n = 3-4$). White bars, 5.5 mmol/l glucose; black bars, 16.5 mmol/l glucose. Glc, glucose; IP, immunoprecipitation; pY, phosphorylated tyrosine. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

on the cell surface. Furthermore, inhibition of IR cleavage by knockdown of putative proteinases enhanced tyrosine phosphorylation of IRS1, but not of IR (Fig. 4h). Collectively, these results indicate that IR cleavage suppresses the insulin signalling pathway via decreased tyrosine phosphorylation of IRS1.

Next, we examined the interaction between the IR and IRS1. The ICD-IR β was found to bind to IRS1, possibly exerting a dominant negative inhibitory effect on tyrosine phosphorylation of IRS1, despite the full length of the IR β subunit binding to IRS1 in a glucose concentration-dependent manner (Fig. 4i). In addition, prolonged exposure to high glucose and metformin affected tyrosine phosphorylation of IRS1 without changing O-glycosylation and serine phosphorylation (Fig. 4j).

Plasma sIR levels correlate with insulin resistance in patients with type 2 diabetes We have previously reported that type 2 diabetic patients exhibited a significantly elevated sIR level compared with controls [6]. The cellular insulin resistance observed in the in vitro model suggests physiological relevance of IR cleavage in vivo. Therefore, we investigated the association between plasma sIR levels and insulin sensitivity (determined using the euglycaemic–hyperinsulinaemic clamp technique) in patients with type 2 diabetes. We found that plasma sIR levels significantly correlated with the glucose clearance rate ($r^2 = 0.374$, $p = 0.012$; Fig. 5).

Discussion

Here, we identified calpain 2 and γ -secretase as proteases that cleave IR and modulate cellular insulin signalling. It is generally accepted that calpain 2 exists and functions in the cytoplasm, but its activity requires a millimolar concentration of calcium to be fully active in vitro [24]. Calpain 2 does not contain a signal peptide which is necessary for the regulated secretory pathway, but nevertheless it has been reported to exist in the extracellular spaces of various tissues [25–27]. Exosomes, a type of extracellular vesicle, are derived from the endosomal pathway. The

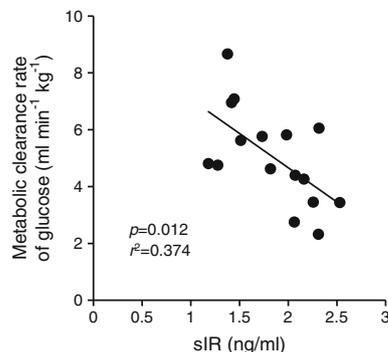


Fig. 5 Plasma sIR levels correlate with insulin resistance in patients with diabetes. The association between sIR levels and insulin sensitivity as assessed by the euglycaemic–hyperinsulinaemic clamp test in patients with type 2 diabetes ($n = 16$)

multivesicular body proceeds to release intraluminal vesicles, such as exosomes, to the extracellular space instead of to the lysosomal degradation pathway [28]. Accumulating evidence shows that exosomes can modulate the cell environment and intracellular communications [29]. Calpain 2 has previously been identified in clathrin-coated vesicles [30], implying that it may be internalised in the endosomal pathway and consequently released into the extracellular space. This study identified for the first time that the extracellular existence of calpain 2 is associated with exosomes.

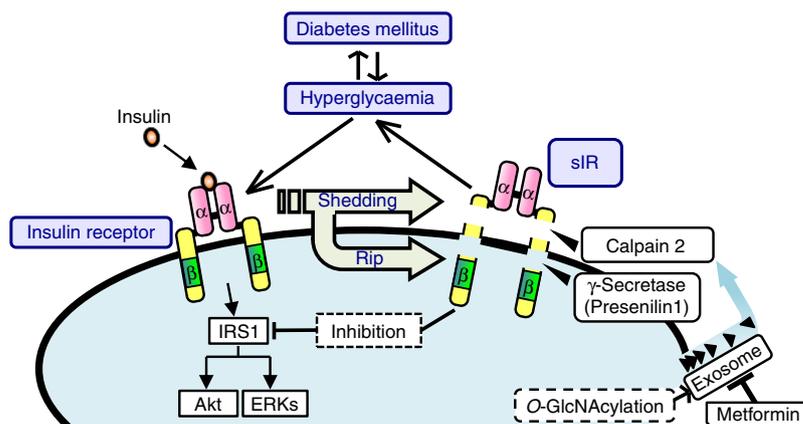
sIR contains a full IR α subunit, therefore its binding to insulin may reduce insulin sensitivity [31]. In the present study, in vitro, Akt signalling was suppressed in high-glucose-treated HepG2 cells, despite washing the cells, changing the medium just prior to insulin treatment and using a ratio of insulin to sIR of approximately $10^5:1$. In the clamp studies, the insulin concentration was about 100 times greater than that of sIR. Nevertheless, higher sIR levels correlated with lower insulin sensitivity. These results indicate that IR cleavage directly disrupts intracellular insulin signalling.

In this study, IR cleavage by calpain 2 and γ -secretase suppressed Akt phosphorylation and reduced tyrosine phosphorylation of IRS1. O-GlcNAcylation and serine phosphorylation of IRS1 have previously been reported to affect the downstream signalling of IRS1 [32, 33]; however, these modifications did not seem to be implicated in the impaired insulin signalling observed in our in vitro assay. Transient overexpression of the recombinant intracellular domain of IR β did not induce a significant decrease in Akt phosphorylation (ESM Fig. 1), because of its lack of binding to IRS1 (ESM Fig. 2). Therefore, tyrosine phosphorylation of ICD-IR β , which has been postulated to be induced by cleavage of the extracellular domain of IR [34], is necessary for its binding to IRS1 and its inhibitory effect on insulin signalling.

IR cleavage potentially suppressed both basal and activated insulin signalling. This finding does not contradict the result that tyrosine phosphorylation of IRS1 was suppressed concomitantly with the increase in IR shedding since IRS1 protein deficiency has previously been reported to suppress basal and insulin-induced Akt activity in primary hepatocytes [35].

The identification of IR cleavage in HepG2 cells and primary hepatocytes indicates that the liver may be an important source of sIR [7]. IRS1 signalling in the liver has been shown to be associated with glucose homeostasis [36]. Systemic insulin resistance observed in *Irs1*-knockout mice was restored by the expression of IRS1 specifically in the liver, indicating that hepatic IRS1 signalling is crucial for systemic insulin sensitivity in mice [35]. In humans, impaired tyrosine phosphorylation of IRS1 in the liver has been suggested to contribute to insulin resistance [37]. Taken together, the clamp study results, showing a significant association between sIR and insulin sensitivity, indicate the possibility that IR cleavage in the liver could potentially induce insulin resistance in patients with type 2 diabetes.

Fig. 6 Schematic representation of the proposed model of IR cleavage in the pathogenesis of diabetes. Rip, regulated intramembrane proteolysis



The molecular mechanism of metformin action is still controversial [38]. Metformin has been found to inhibit mitochondrial respiratory chain complex I, consequently increasing the AMP:ATP ratio, leading to activation of AMP-activated protein kinase (AMPK) and a reduction in gluconeogenic gene transcription [39]. In our model, the AMPK inhibitor compound C did not reduce the effects of metformin (ESM Fig. 3), indicating that it is unlikely that metformin suppresses IR cleavage via AMPK. Metformin has also been reported to decrease blood glucose levels independently of AMPK and liver kinase B1 [40]. Metformin has been shown to inhibit both adenylate cyclase, which induces suppression of glucagon signalling in hepatocytes [41], and mitochondrial glycerophosphate dehydrogenase, which suppresses endogenous glucose production [42]. It has also been shown to enhance insulin signalling through inhibition of the mechanistic target of rapamycin complex 1 (mTORC1) pathway [43]. The phosphorylation levels of mTOR and p70 S6 kinase (p70S6K) remained unchanged in response to high glucose or metformin treatment, indicating that the recovery of tyrosine phosphorylation of IRS by metformin is independent of mTORC1 signalling (ESM Fig. 4).

This study indicates that metformin prevents exosome release. Previous studies have reported that p53 induces tumour suppressor-activated pathway 6 (TSAP6) expression, which has been shown to increase exosome release [44], and that metformin decreases p53 expression in HepG2 cells [45]. In our model, no increments of p53 and TSAP6 were observed with high glucose, and metformin did not change the expression of these molecules (ESM Fig. 5), indicating that it is unlikely that metformin suppresses calpain 2 release through the p53–TSAP6 pathway. Although further study is necessary to identify the mechanism by which metformin regulates exosome-associated calpain 2 release, inhibition of IR cleavage offers a novel insulin-sensitising mechanism that is involved in the glucose-lowering effects of metformin.

Our findings, which imply that IR cleavage by a calcium-dependent protease, calpain 2, modulates insulin signalling in vitro and insulin resistance in vivo, may be relevant to the molecular mechanism of chelation therapy using EDTA.

Chelation therapy in humans is known to improve insulin sensitivity and induce hypoglycaemia [46], effects that can be reversed by a calcium injection [47]. This observation could be explained by the results presented here and not by any conventional theories related to insulin sensitivity.

In summary, we have shown that exosome-associated calpain 2 cleaves IR extracellularly, and that sequential cleavage of IR by γ -secretase impairs the phosphorylation cascade of insulin signalling. This effect was ameliorated by metformin in HepG2 cells. The existence of sIR in human blood strongly suggests that IR cleavage modulates insulin signalling in vivo, whilst findings from the clamp study indicate that IR cleavage is associated with peripheral insulin resistance in patients with diabetes. These findings may establish a new framework in which IR cleavage regulates glucose homeostasis. The molecular mechanism underlying IR cleavage may help to explain the pathogenesis of insulin resistance and provide a new therapeutic target for type 2 diabetes (Fig. 6).

Acknowledgements We thank: Y. Ebina (Tokushima University), for his advice and discussion, T. Saitoh (Tokushima University), for help with conducting the study and K. Sakai (Tokushima University), for her technical assistance. We also thank Edanz Group Japan (Fukuoka, Japan) for help with revising the language of the manuscript.

Funding This work was supported by the Japan Society for the Promotion of Science KAKENHI, grant numbers 22790317, 24790317, 15K09391 (to TY), 25870483 (to KA-S) and 24790929 (to SI).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement TY was responsible for conceptualising the study, data collection, analysis and interpretation, writing the manuscript and the integrity of the whole work. KA-S, SI and SH contributed to data collection, analysis and the writing of the manuscript concerning the in vitro study. AK and MM contributed to data collection, analysis, interpretation and the writing of the manuscript concerning the clinical study. MT, TM and HK contributed to data interpretation and the writing of the manuscript concerning the clinical study. All of the authors approved the final version of the manuscript. TY is the guarantor of this work.

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