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Development of *in vitro* model of insulin receptor cleavage induced by high glucose in HepG2 cells

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ABSTRACT

Soluble insulin receptor (sIR), the ectodomain of IR, has been detected in human plasma, and its concentration parallels that of blood glucose in patients with diabetes. IR has a pivotal role in glucose homeostasis and diabetes development; therefore, cleavage of IR promoted by hyperglycemia is involved in insulin resistance and glucose toxicity. To elucidate the physiology of sIR, we developed an *in vitro* model mimicking the changes in sIR levels in plasma from patients with diabetes. Among four human cell lines that expressed IR, spontaneous cleavage of IR occurred only in HepG2 cells. The molecular characteristics of sIR derived from HepG2 cells were similar to those of sIR detected in human plasma. The concentration of sIR in the medium did not differ between basal and high-glucose conditions in the initial 24-h period, but increasing the duration of pre-stimulation (>48 h) led to a significant increase in sIR levels in cells exposed to high glucose. Additionally, glucose-dependent increment of sIR was reversible in this model. These results are consistent with the observation of plasma sIR in patients with diabetes. Using this model, O-linked N-acetylglucosamine modification was determined to be involved in high-glucose-induced IR cleavage. A calcium-dependent protease was shown to cleave IR extracellularly. These findings show that this *in vitro* model could be useful for determining the molecular mechanism underlying IR cleavage.

1. Introduction

Insulin receptor (IR) exists as a heterotetramer on the plasma membrane. The α subunit, which binds insulin, and the β subunit, which contains the tyrosine kinase domain, are linked by disulfide bonds [1]. Insulin binds to its receptor and activates tyrosine kinase, which consequently initiates the intracellular signaling pathway. We found that soluble insulin receptor (sIR), the IR ectodomain, exists in human plasma samples. Unexpectedly, plasma sIR levels were significantly higher in patients with diabetes than in the control group. Among several metabolic disorders associated with diabetes, significant positive correlations were found between sIR and fasting blood glucose or hemoglobin A1c [2]. Although hyperglycemia is thought to be responsible for elevated plasma sIR levels, the molecular mechanisms of sIR generation remain unclear.

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 [3]. Here, we present the first *in vitro* model using HepG2 liver-derived cells, which describes the molecular biology of IR cleavage generating sIR.

2. Materials and methods

2.1. Materials

Deoxynorleucine (DON), O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenyl carbamate (PUGNAc), and L-685,458 were purchased from Sigma–Aldrich (St. Louis, MO,

Abbreviations: ADAM, a disintegrin and metalloprotease; BAPTA-AM, O,O′-Bis [2-aminophenyl] ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester; DON, deoxynorleucine; ICT-EIA, immune complex transfer enzyme immunoassay; IR, insulin receptor; OGA, β-N-acetylglucosaminase; O-GlcNAc, O-linked N-acetylglucosamine; OGT, O-linked-N-acetylglucosaminyltransferase; PNGase F, peptide: N glycosidase F; PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenyl carbamate; MMP, matrix metalloproteinase; sIR, soluble insulin receptor.

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USA). Peptide: N-glycosidase (PNGase) F was purchased from New England Biolabs (Ipswich, MA, USA). MG132 was purchased from Merck-Millipore (Billerica, MA, USA). O,O′-Bis [2-aminophenyl] ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) was purchased from Dojindo (Kumamoto, Japan). The specific antibodies used are listed in Supplemental data. All other reagents used were of analytical grade.

2.2. Cell culture

HeLa, HEK293, A549, and HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) basal glucose (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS). Human primary hepatocytes and human umbilical vein endothelial cells (HUVEC) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured according to the manufacturer's instructions. All cells were cultured at 37 °C in 5% CO_2. For the IR cleavage assay, HepG2 cells were seeded at a density of 3×10^5 cells/well on 24-well plates coated with collagen type I (IWAKI, Tokyo, Japan). This density was determined to be the most appropriate for high-glucose-induced IR cleavage (data not shown). Starting from 24 h after seeding, the medium containing various glucose concentrations plus 1% FBS was replaced every 24 h. A similar experimental protocol using the prolonged high-glucose treatment was shown not to cause apoptosis in HepG2 cells [4].

2.3. Ultrasensitive ELISA for human sIR

We used an ultrasensitive enzyme-linked immunoabsorbent assay (ELISA), immune complex transfer enzyme immunoassay (ICT-EIA), to measure the levels of human sIR as previously described [5]. In brief, the immune complexes of sIR with antibodies are transferred from a solid phase to another solid phase. Nonspecific signals are reduced by the transfer, thereby this method can measure sIR at levels of 0.004 pg, with a sensitivity of 0.04 pg/ml. Two kinds of monoclonal antibodies against human specific IR α subunit, 5D9 and 83-7, were used. Neither of these antibodies bound to the insulin-like growth factor 1 receptor (IGF1-R) [6,7]. Our investigations indicate that this assay is not able to detect for the IR/IGF1-R hybrid receptors. Ultracentrifugation (100,000×*g* for 1 h) of the medium did not diminish the sIR increment promoted by high-glucose treatment, indicating that sIR (excluding IR on plasma membrane) was evaluated in these experiments. 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. We developed a new high-throughput ultrasensitive immunoassay method based on the ICT-EIA for human sIR (unpublished data).

2.4. Gel filtration assay

HepG2 cell medium (0.8 ml) and purified recombinant human IR ectodomain diluted in DMEM containing 1% FBS were applied to a Superose 6 gel-filtration column (Sigma–Aldrich) on an AKTA FPLC system (GE Healthcare, Little Chalfont, UK). The sIR (pg/ml) titer in each fraction (0.5 ml) obtained from either HepG2 medium or purified recombinant human IR ectodomain was determined by the ICT-EIA for sIR. The approximate molecular weight markers (Gel Filtration Calibration Kit HMW; GE Healthcare) were also used to demonstrate calibration of the column.

2.5. Immunodepletion assay

HepG2 cells were incubated with 16.5 mM glucose in DMEM containing 1% FBS for 96 h. Equal amounts of cell lysates and medium were subjected to the immunodepletion assay. The specific antibodies conjugated to Protein A–Sepharose CL-4B beads (GE Healthcare) were added and rotated at 4 °C for 16 h. After the Sepharose beads were spun down, the supernatants were subjected to the ICT-EIA for sIR.

2.6. Immunoprecipitations and Western blot analysis

Equal amounts of protein were subjected to immunoprecipitation with specific antibodies, and Western blot analysis was done as previously described [8]. In each Western blot analysis, a representative experiment was shown.

2.7. siRNA knockdown

Dharmacon siRNAs for human O-linked-N-acetylglucosaminyltransferase (OGT) (L-019111-00-0005) and β-N-acetylglucosaminase (OGA) (L-012805-00-0005), as well as the control siRNA (D-001810-10-05), were purchased from Thermo Scientific (Waltham, MA, USA). HepG2 cells were seeded at a density of 3×10^5 cells/well on 24-well plates coated with collagen type I. The cells were transfected with the indicated siRNAs using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocol. At 24 h after transfection, the medium was replaced every 24 h with DMEM containing 5.5 or 16.5 mM glucose plus 1% FBS.

2.8. Statistical analysis

Values are represented as mean ± SEM. Differences between two groups were analyzed with an unpaired *t* test. Data involving more than two groups were analyzed with one-way of ANOVA, and the multi-comparison test was adjusted using Bonferroni's corrections with significance level of 0.05, 0.01 or 0.001.

3. Results

3.1. HepG2 cells generate sIR

The concentration of sIR in the medium of human cell lines was too low to be accurately measured by ELISA kits specific for human plasma sIR [2]. Therefore, we developed an ultrasensitive ELISA for human sIR [5]. Using this system, human primary hepatocytes and HUVEC were shown to generate sIR. We also found that of four human cell lines that expressed IR, spontaneous cleavage of IR occurred only in HepG2 cells, resulting in the appearance of sIR in the medium (Fig. 1A). The generation of sIR in HepG2 cells was correlated with the duration of cell culture (Fig. 1B). It has been previously reported that HepG2 cells produce the IR/IGF1-R hybrid receptors [9], and the abundance of these receptors is increased in skeletal muscle of patients with diabetes [10]. However, our ELISA system does not cross-react with these hybrid receptors (see Section 2).

The molecular characteristics of sIR derived from HepG2 cells were determined. The retention time of sIR was established by gel-filtration chromatography and was identical to that of recombinant human IR ectodomain (Fig. 1C). The concentration of residual sIR was determined in the medium after immunodepletion using several anti-IR antibodies. The antibody 5D9 recognizing epitope on the IRα subunit and the antibody 18–44 recognizing epitope on the N-terminal of the IRβ subunit depleted most of the sIR from the medium. By contrast, the antibody C-19 recognizing

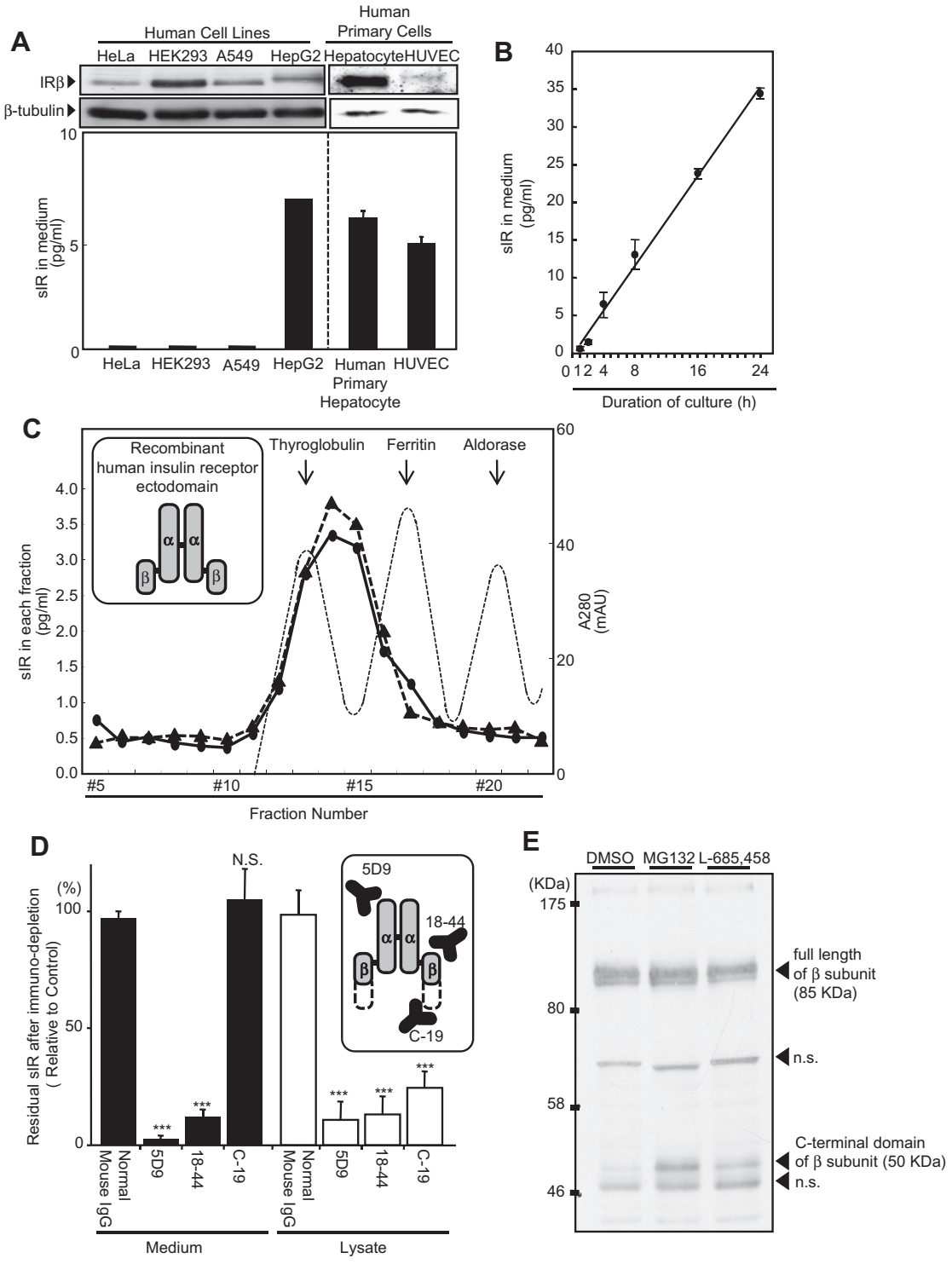

Fig. 1. IR is cleaved in primary human cells and HepG2 Cells. (A) sIR level in the medium was measured after incubating each cell for 24 h ($n = 2$). Representative Western blots show protein expression of IR in each human cell. (B) Graph shows the sIR level in the medium incubating HepG2 cells for the indicated times. (C) sIR titer in each fraction obtained from either HepG2 medium (filled circles) or purified recombinant human IR ectodomain (filled triangles). (D) Graph shows the amount of residual sIR in the supernatants after immunodepletion using the indicated antibodies relative to that of the control (Sepharose alone) ($n = 4$). ***, N.S.: versus each normal mouse IgG. (E) HepG2 cells were incubated for 72 h. Cell lysates were prepared after incubating HepG2 cells in dimethyl sulfoxide, 5 μM MG132, or 4 μM L-658,485 for an additional 24 h. C-terminal domain of β subunit indicates two kinds of truncated IRβ subunit: (MG132) intramembrane-cleaved IRβ subunit generated by γ-secretase-regulated intramembrane proteolysis, and (L-685,458) intracellular domain of IRβ subunit generated by ectodomain shedding. n.s.: non-specific band.

epitope on the C-terminal of the IRβ subunit did not deplete the sIR levels (Fig. 1D). These results indicate that sIR derived from HepG2 retains the N terminus but not the C terminus of the IRβ subunit. Plasma sIR was also depleted by the antibodies 5D9 and 18–44, but not by C-19 (data not shown). Taken together, sIR derived from HepG2 showed similar molecular characteristics to those of sIR detected in human plasma and recombinant human IR ectodomain [2]. The C-terminal domain of the IRβ subunit was accumulated by inhibiting proteosomal degradation (MG132) or γ-secretase activity (L-658,485) (Fig. 1E), indicating that ectodomain shedding

of IR precedes γ-secretase cleavage, and then the intramembrane-cleaved IRβ subunit undergoes rapid proteasomal degradation, as previously reported [11].

3.2. IR is cleaved in a glucose-dependent manner

The experimental conditions responsible for high-glucose-induced release of sIR into the medium were investigated. The medium was replaced every 24 h. The concentration of sIR was measured in the last 24-h period after pre-stimulation with basal (5.5 mM) or high (16.5 mM) glucose at various times (Fig. 2A, upper panel). We found no difference in sIR concentration between basal and high-glucose conditions in the initial 24-h period, but increasing the duration of pre-stimulation significantly increased sIR levels in cells exposed to high glucose (Fig. 2A, lower left panel). In this model, the amount of IR in whole-cell lysates of HepG2 cells increased in accordance with the duration of pre-stimulation (Fig. 2A, lower middle panel). The net increase in activity of IR cleavage was determined by calculating the ratio of the amount of sIR in the medium to that of IR in the whole-cell lysate. Following pre-stimulation of HepG2 cells for >48 h, the concentration of sIR was significantly greater in cells exposed to a high compared with basal glucose level (Fig. 2A, lower right panel). These results are consistent with the observation that it takes several days for plasma sIR levels in patients to change following changes in glucose levels [2].

The reversibility of IR cleavage induced by high glucose levels was confirmed in this model. After treating HepG2 cells with basal (5.5 mM) or high (11 mM, instead of 16.5 mM owing to exfoliation of HepG2 cells in the last period) glucose for 72 h, the medium was replaced with a basal glucose medium every 24 h (Fig. 2B, left panel). After replacing high glucose with a basal glucose level, the medium collected in the first 24-h period had a high concentration of sIR. However, the amount of sIR gradually decreased with increasing duration of basal glucose culture (Fig. 2B, right panel). These results indicate that glucose-dependent IR cleavage in this model is reversible, as was also observed in patients with diabetes [2].

To investigate the effect of high osmolarity in the medium on IR cleavage, sorbitol, mannitol and sodium chloride were added into 5.5 mM glucose medium to adjust the osmolarity of the solutions to 16.5 mOsmol/kg. The concentration of sIR was not increased by the high osmolarity in the medium (Fig. 2C).

3.3. O-GlcNAcylation is responsible for IR cleavage

A further investigation was done as to whether O-linked N-acetylglucosamine (O-GlcNAc) modification was involved in IR cleavage (Fig. 3A). DON, which acts as a glutamine: fructose-6-phosphate amidotransferase antagonist and decreases O-GlcNAc moieties [12], suppressed high-glucose-induced generation of sIR (Fig. 3B). By contrast, PUGNAc, which acts as an antagonist against OGA and inhibits the enzymatic removal of the O-GlcNAc modification [13], increased the amount of sIR as compared with untreated cells, even in the basal glucose medium. The amount of IR in each experimental condition was unchanged (Fig. 3C). Knockdown of OGT or OGA using appropriate siRNAs for each gene decreased and increased O-GlcNAc modification, respectively (Fig. 3D). OGT knockdown reduced high-glucose-induced IR cleavage, whereas OGA knockdown significantly increased sIR levels (Fig. 3E), without affecting the amount of IRβ (Fig. 3D). The α and β subunits of IR were both shown to be mediated by O-GlcNAc modification. However, these modification levels were not altered, even under high-glucose conditions (Fig. 3F).

As shown in Fig. 1A, the molecular weight of IR in HepG2 cells seems to be greater than that of other human cell lines. An *in vitro* deglycosylation assay using PNGase F showed that N-glycosylation was responsible for the differences in the molecular weight of the IR between HepG2 cells and other human cell lines (Fig. 3G).

3.4. Calcium-ion-dependent proteinase cleaves IR extracellularly

Matrix metalloproteinase (MMP) and ADAM (a disintegrin and metalloprotease) are thought to be potential proteases involved in IR cleavage, because these metallopeptidases are reported to cleave various membrane proteins [14,15]. These proteases require a metal ion [16]; therefore, we used metal chelators of divalent cations to examine their involvement in IR cleavage. Treatment for 1 h with EDTA and EGTA (to minimize the effect on intracellular calcium), which are frequently used to block the activity of these proteases, significantly decreased the amount of sIR in the medium (Fig. 4A), indicating that metal-dependent proteases are involved in IR cleavage. The inhibitory effects of EGTA were restored by the addition of calcium ions (Fig. 4B). The addition of calcium ions to the buffer increased the generation of sIR, whereas other metal ions (iron or magnesium) included in DMEM did not (Fig. 4C). The calcium ions increased the concentration of sIR in a sigmoid manner (Fig. 4D), suggesting that the putative protease involved in IR cleavage is allosterically regulated by calcium ions. The proteolytic effects of high-glucose and PUGNAc on IR cleavage were inhibited by EGTA without changes of IRβ abundance in cells (Fig. 4E). By contrast, BAPTA-AM, which chelates intracellular calcium ions (Fig. 4F), and ionomycin, which increases intracellular calcium ions (Fig. S1), did not affect sIR levels in the medium.

4. Discussion

This investigation describes an *in vitro* model of sIR using HepG2 cells in which IR underwent a proteolytic process. As shown in Fig. 1B, the IR was spontaneously cleaved, even under basal glucose conditions. During IR isolation from the human placenta, the truncated β subunit, which is derived from the extracellular portion of the β subunit by removing an intracellular C-terminal fragment, has been detected [1], suggesting that the IRβ subunit includes the target domain for proteolytic cleavage just above the cell membrane. In addition, proteasome and γ-secretase inhibitors induced the accumulation of an additional fragment with a molecular weight of around 50 kDa, which appears to be the C-terminal domain of the IRβ subunit (Fig. 1E). The abundance of this domain of IR increased in proportion to the generation of sIR following exposure to a high glucose concentration (Fig. S2). Therefore, in our model the IR was likely to have undergone the first proteolysis on the cell membrane, and the second proteolysis occurred in the transmembrane domain. This observation is consistent with a mechanism that regulated intramembrane proteolysis by γ-secretase is preceded and regulated by an initial distinct cleavage in ectodomain shedding [17].

In terms of the biochemical mechanisms governing the clinical features of glucose-dependent sIR generation, O-GlcNAc modification was shown to be involved in IR cleavage (Fig. 3B–E). Approximately 2–3% of cellular glucose enters the hexosamine biosynthesis pathway to generate molecules such as UDP-GlcNAc, which are used as the moieties of O-linked glycosylation [18]. Elevated O-GlcNAc modification of proteins has been reported to develop insulin resistance; however, the causal relationship has not been established [19]. We observed that exposing HepG2 cells to high glucose did not alter the whole concentration of O-GlcNAc modification (Fig. 3D), but rather that of specific proteins that are likely to be responsible for the increase of IR cleavage. The O-GlcNAc modification levels of IR remained unchanged under high glucose concentrations (Fig. 3F), indicating that the O-glycosylated IR

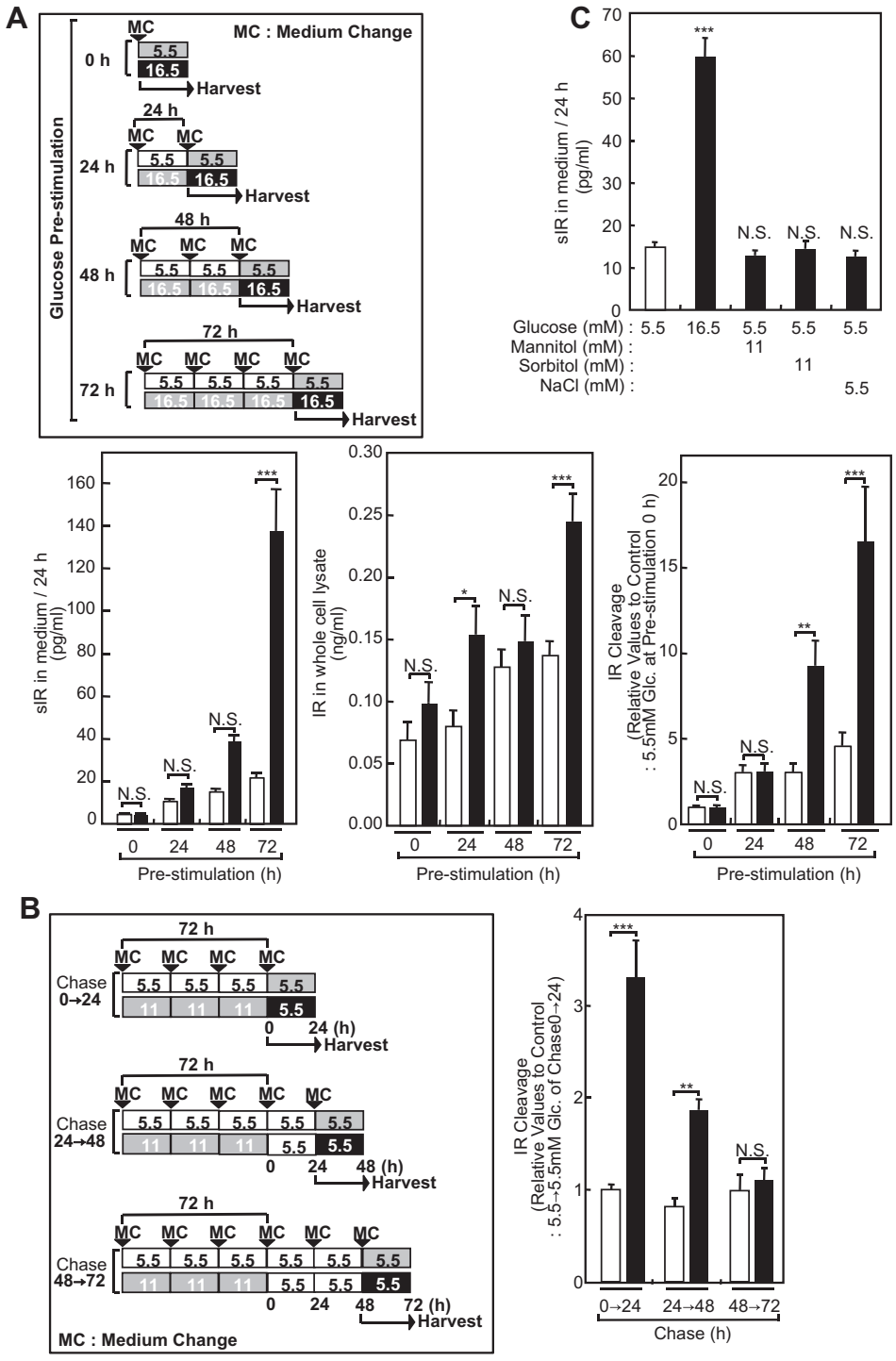

Fig. 2. IR cleavage in HepG2 cells is dependent on glucose concentration in the medium. (A) Protocol for assessing high-glucose-induced IR cleavage (upper panel). HepG2 cells were incubated with 5.5 mM (white bar) or 16.5 mM (black bar) glucose (glucose pre-stimulation). Graphs show sIR levels in the medium for an additional 24 h (lower left panel), IR levels in cell lysates (lower middle panel), and net increase in IR cleavage, relative to that in control cells (lower right panel) ($n = 12$). (B) Protocol for assessing the reversibility of high-glucose-induced IR cleavage (left panel). HepG2 cells were incubated with indicated glucose concentrations for 72 h, after which the medium was replaced with 5.5 mM glucose for 0, 24, or 48 h. Graph shows the net increase in IR cleavage determined by cells incubated for an additional 24 h ($n = 9$). White bar, 5.5 → 5.5 mM glucose; black bar, 11 → 5.5 mM glucose (right panel). N.S.: not significant. (C) HepG2 cells were incubated with an osmolarity (16.5 mOsmol/kg) identical to that of 16.5 mM glucose for 72 h. Graph shows the sIR level in the medium incubating cells for an additional 24 h ($n = 9$). ***, N.S.: versus 5.5 mM glucose.

is not implicated in changes of IR cleavage. Further studies using this model are necessary to identify the target molecules of O-GlcNAcylation.

The putative membrane protease assigned to cleave the IR was observed to be dependent on calcium ions (Fig. 4B–D). Neither intracellular calcium chelator (BAPTA-AM) nor calcium ionophore (ionomycin) influenced the IR cleavage (Figs. 4F and S1), indicating that the proteolysis of IR occurs in the extracellular space. Some MMPs have been reported to digest the IRα subunit at some positions [20]. However, neither the zinc ion chelator nor the broad MMP/ADAM inhibitors diminished high glucose-induced proteolytic release of sIR (Fig. S3), suggesting that MMPs and ADAMs are not candidates for IR cleavage in our model. Chelation with EDTA *in vivo* is known to cause hypoglycemia, which can be

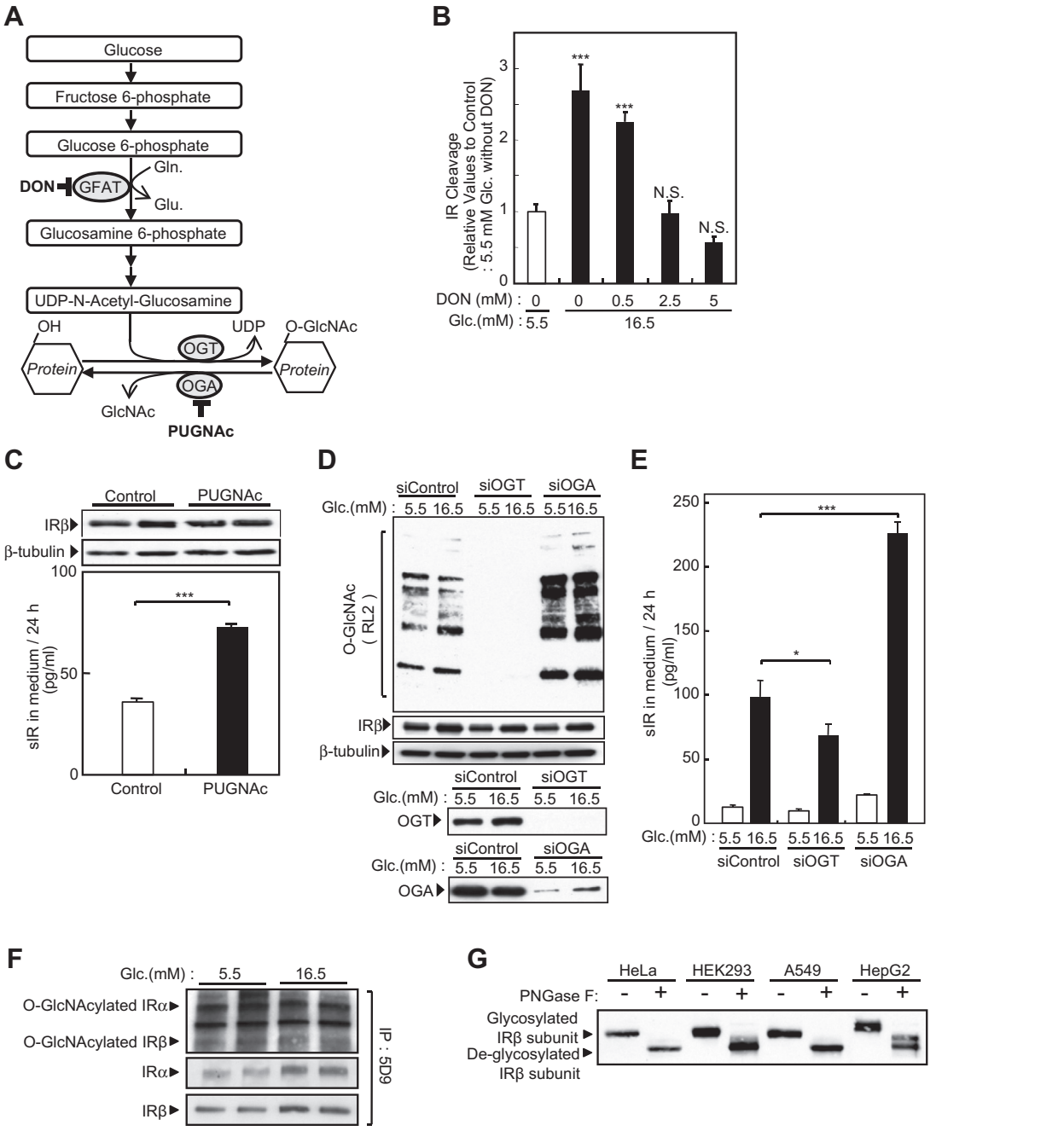

Fig. 3. O-GlcNAcylation facilitates IR cleavage. (A) Simplified schematic representation of the hexosamine biosynthesis pathway and O-GlcNAcylation. (B) HepG2 cells incubated with DON for 72 h. Graph shows the net increase in IR cleavage determined by HepG2 cells incubating for an additional 24 h (n = 7–9). ***, N.S.: versus control condition (5.5 mM glucose without DON). (C) HepG2 cells were incubated with control (1% ethanol) or 100 μM PUGNAc in DMEM containing 5.5 mM glucose for 72 h (n = 9). The medium and cell lysate were prepared after incubating cells for an additional 24 h. (D and E). HepG2 cells were transfected with siRNAs (10 nM) and incubated for 72 h. The medium (D) (n = 9) and cell lysate (E) were prepared after incubating HepG2 cells for an additional 24 h. (F) HepG2 cells were incubated with 5.5 or 16.5 mM glucose for 96 h. O-GlcNAcylated subunits of IR were shown after immunoprecipitation with 5D9. (G) Equal amounts of each cell lysate were incubated *in vitro* with PNGase F at 37 °C for 24 h, and were subjected to Western blotting using anti-IRβ subunit antibodies.

ameliorated by administrating a calcium injection [21]. These observations suggest that calcium-ion-dependent IR cleavage may play an important role in glucose homeostasis.

We also found that by the clamp technique, plasma sIR levels were shown to be inversely correlated with insulin sensitivity in patients with diabetes, and metformin prevented IR cleavage in our *in vitro* model [22]. These observations suggest that IR cleavage is a possible component of insulin resistance induced by high glucose.

In the present study, we described an *in vitro* model using HepG2 cells, which mimicked the changes in sIR levels in plasma from patients with diabetes. Using this model, O-linked N-acetylglucosamine modification and calcium-dependent protease were shown to be involved in IR cleavage. This model could be useful for determining the molecular mechanism underlying IR cleavage.

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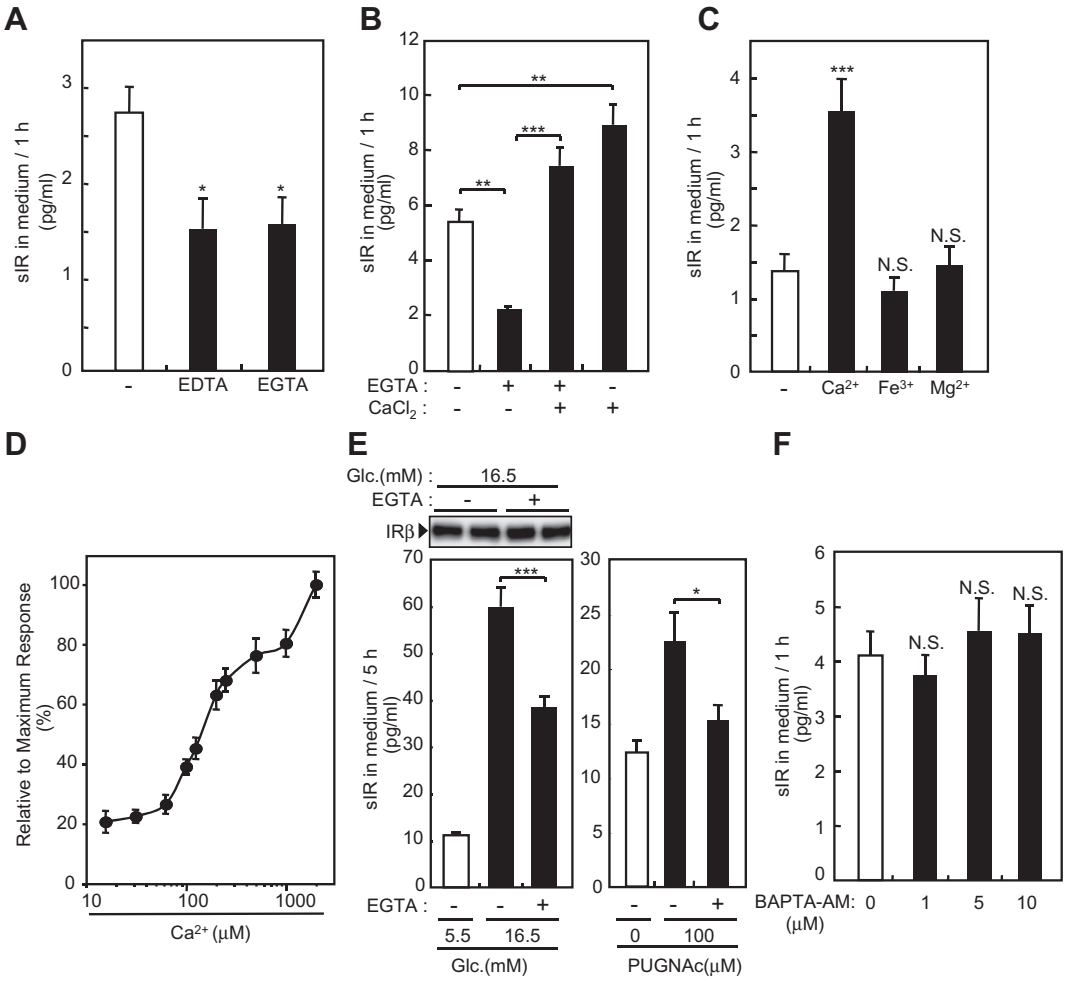

Fig. 4. IR cleavage is dependent on calcium ions. (A) HepG2 cells were incubated with 16.5 mM glucose for 72 h. Graph shows sIR levels for a further 1 h in serum-free DMEM containing 5 mM EDTA or 5 mM EGTA (n = 9–10). (B–D) HepG2 cells were incubated with 16.5 mM glucose for 72 h. Graphs show (B) sIR levels for a further 1 h in serum-free DMEM containing 5 mM EGTA and/or 5 mM calcium chloride (n = 6), (C) those in HEPES-buffered saline containing 2 mM calcium chloride, 0.5 μM iron (III) chloride, or 1 mM magnesium sulfate (n = 9), ***, N.S.: versus control condition, (D) response relative to the maximum response in HEPES-buffered saline containing the indicated concentration of calcium for a further 1 h. (E) HepG2 cells were incubated with 5.5 or 16.5 mM glucose (left panel) or 5.5 mM glucose and 100 μM PUGNAc (right panel) for 72 h. The medium and cell lysate were prepared after incubating cells with or without 5 mM EGTA for a further 5 h (n = 9). (F) HepG2 cells were incubated with 16.5 mM glucose for 72 h. Graph shows sIR levels in serum-free DMEM containing the indicated concentration of BAPTA-AM for a further 1 h (n = 8–9). N.S.: versus control condition.

Conflict of interest

No potential conflicts of interest relevant to this article are reported.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.187.

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