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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