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Stratified analysis of lectin-like chaperones in the folding disease-related metabolic syndrome rat model

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ABSTRACT

The metabolic syndrome including obesity and diabetes mellitus is known to be a major health problem worldwide. A recent study reported that obesity causes endoplasmic reticulum (ER) stress and subsequently leads to insulin resistance and type 2 diabetes. However, little is known about the alterations in the components of the calnexin/calreticulin (CNX/CRT) cycle, which promote glycoprotein folding in obese and diabetic conditions. To understand the operating status of the lectin-like chaperones related to the CNX/CRT cycle in the metabolic syndrome, we analyzed the chaperones for the activity, protein expression, and mRNA expression levels using Zucker fatty (ZF) and Zucker diabetic fatty (ZDF) rat models for obesity and diabetes, respectively. We demonstrated that misfolded proteins were gradually increased with progression of the syndrome, obesity to diabetes. The individual chaperone activities of CNX and CRT were both decreased in the ZF rat ER and, in contrast, were increased in the ZDF rat ER. The protein quantities and mRNA expressions of CNX and CRT were decreased in the ZF rats, but increased in the ZDF rats compared with those of the healthy model. Therefore, these results indicate that obesity down-regulates CNX and CRT expressions and their activities and diabetes up-regulates the expressions and activities of CNX and CRT. Our findings clearly suggest that metabolic syndrome affects the lectin-like chaperones in the CNX/CRT cycle at both the activity and expression levels.

1. Introduction

Obesity and diabetes mellitus are components of metabolic syndrome. Obesity promotes insulin resistance, resulting in the development of diabetes. Although the mechanisms have remained unclear for a long time, Özcan et al. [1] reported that obesity causes endoplasmic reticulum (ER) stress, which subsequently leads to insulin resistance and type 2 diabetes. The mechanism involves insulin receptor signaling, which is suppressed through hyperactivation of c-Jun N-terminal kinase and subsequent phosphorylation of insulin receptor substrate-1. In addition, the deficiency of the transcription factor X-box-binding protein-1 modulates the ER stress response and induces insulin resistance [1]. Thus, these pathophysiological conditions also alter the lectin-like chaperones related to the calnexin/calreticulin (CNX/CRT) cycle that promotes glycoprotein folding [2–5]. However, little is known about the alterations in the cycle components under obese and diabetic conditions. In the cycle, CNX and CRT recognize nascent glycoproteins with monoglucosylated high-mannose type *N*-glycan and assists in folding cooperatively with ERp57, a multifunctional thiol-disulfide oxidoreductase (EC 5.3.4.1) [6,7].

We investigated the effects of obesity and diabetes on the lectin-like chaperones, CNX and CRT, using the Zucker fatty (ZF) and the Zucker diabetic fatty (ZDF) rats as the obesity and diabetes models, respectively. The ZF rat, which appeared spontaneously in the 13 M rat stock [8], has been used as an obesity animal model, associated with genetic leptin receptor deficiency ($Lepr^{fa/fa}$), leading to high blood lipid levels and increased body weight [9,10]. The ZDF rat, an inbred strain of the ZF rat that presents with type 2 diabetes [11], is characterized by hyperglycemia, hyperinsulinemia, hyperlipidemia, hypertension, and obesity [12]. Accordingly, these model rats originated from the same strain and were suitable for investigating the gradual effects of metabolic syndrome on CNX and CRT.

To understand the gradual changes in the operating status of the lectin-like chaperones related to the CNX/CRT cycle in the metabolic syndrome, which concerns the folding diseases, we analyzed

Abbreviations: CRT, calreticulin; CNX, calnexin; d-OVA, denatured ovalbumin; ER, endoplasmic reticulum; MDH, malate dehydrogenase; OVA, ovalbumin; TBS, Tris-buffered saline; ZDF, Zucker diabetic fatty; ZF, Zucker fatty; ZN, Zucker normal.

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the operating status of the chaperones at the activity, protein expression, and mRNA expression levels.

2. Materials and methods

2.1. Materials

The liver tissues of ZF and Zucker normal (ZN) rats (male, 10 weeks old) representing obesity and the age-matched control models, and ZDF and ZF rats (male, 12 weeks old) representing diabetes and the age-matched control models were purchased from Sankyo Labo Service (Tokyo, Japan) and Charles River Laboratories Japan (Yokohama, Japan). The antibodies were obtained from the indicated sources: rabbit anti-CRT monoclonal antibody (EPR3924) from Abcam (Cambridge, UK), rabbit anti-CNX (H-70) polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit anti-β-actin polyclonal antibody from Cell Signaling Technology (Beverly, MA, USA), rabbit anti-GRP78/BiP polyclonal antibody from Abcam, mouse anti-GM130 monoclonal antibody (35/GM130) from BD Transduction Laboratories (San Jose, CA, USA), HRP-labeled goat anti-rabbit IgG antibody from Perkin Elmer (Waltham, MA, USA), and HRP-labeled goat anti-mouse IgG antibody from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of tissue homogenate and ER-enrichment fraction

Rat liver (0.5 g) was washed with PBS (pH 7.4) and homogenized in 2 mL of an extraction buffer (50 mM HEPES [pH 7.8]/1.25 M sucrose/5 mM EGTA/125 mM KCl) containing an EDTA-free protease inhibitor cocktail using a Potter–Elvehjem homogenizer at 4 °C. The homogenate was centrifuged (1000 × *g*, 10 min, 4 °C) to remove cell debris and nuclei. The resulting supernatant was used as the homogenate for the following protein expression analysis and for preparation of the ER-enrichment fraction. ER-enrichment fraction (rough ER) was prepared using an Endoplasmic Reticulum Isolation Kit (Sigma-Aldrich), which is a combination method of centrifugations and Ca^{2+} precipitation, according to the manufacturer's instructions. The quality was assessed with western blot analyses of BiP and GM130 as ER and Golgi apparatus markers, respectively (Fig. S1). The ER-enrichment fraction (4 mg protein/mL) was homogenized in a buffer (50 mM HEPES [pH 7.4]/2% Triton X-100) and centrifuged (105,000 × *g*, 1 h, 4 °C) to obtain solubilized ER proteins. Solubilized ER proteins were used for total and individual chaperone activity analyses.

2.3. Quantity of misfolded proteins in the ER fractions

The ER-enrichment fraction (0.3 mg protein/mL) was mixed with 5000× concentration of SYPRO Orange dye (Life Technologies; Carlsbad, CA, USA) at a final concentration of 60×, which could specifically bind with the hydrophobic regions on proteins and then emit fluorescence depending on the surface hydrophobicity of the proteins. The fluorescence intensity was measured using a FlexStation3 spectrofluorometer (Molecular Devices; Sunnyvale, CA, USA).

2.4. Total chaperone activity analysis

Malate dehydrogenase (MDH; EC 1.1.1.37) (Oriental Yeast Co., Ltd, Tokyo, Japan) was conjugated with Cy5 using a Cy5-labeling kit (GE Healthcare; Amersham Place, MO, USA). MDH-Cy5 (0.1 μM) denatured by heating at 100 °C for 10 min was mixed with 1 mg protein/mL of ER-enrichment fraction, as the chaperone source in a buffer (10 mM HEPES [pH 7.4]/10 mM $CaCl_2$/0.6% Triton X-100), and preincubated at 4 °C for 24 h to promote MDH-chaperone interaction. Then bovine trypsin (2 μg/mL) was added to the mixture and incubated at 30 °C for 30 min to degrade MDH-Cy5 unbound with chaperone proteins. The mixture including intact MDH-Cy5 and degraded MDH-Cy5 was resolved with tricine-SDS-PAGE (16% gel concentration for separation gel). The electrophoresed gels were analyzed with a FluorChemQ image analyzer (Proteinsimple; San Jose, CA, USA) to detect Cy5 fluorescence.

2.5. CRT/CNX activity analysis

Chaperone activities of CRT and CNX were analyzed with the denatured ovalbumin (d-OVA)-conjugated column method as described previously [13]. The solubilized ER proteins (20 μg in 30 μL) were subjected to chromatography on a d-OVA-conjugated column (5.5 mm × 42 mm, 1 mL of beads, 65 nmol of OVA) equilibrated with TBS (pH 7.4)/10 mM $CaCl_2$/0.6% Triton X-100, at a flow rate of 20 μL/min at 4 °C. The eluted fractions (60 μL) were analyzed using western blotting of CRT and CNX as described in Section 2.6. Bands were analyzed using the FluorchemQ image analyzer to determine the 50% elution volumes of CRT and CNX.

2.6. Quantitative analysis of protein expression level in rat liver homogenate

The rat liver homogenates were resolved on SDS-PAGE (10% Tris-HCl gel) with reducing conditions and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were treated with anti-CRT (5000-fold dilution), CNX (1000-fold dilution), or β-actin (1000-fold dilution), and subsequently with HRP-conjugated anti-rabbit IgG (10,000-fold dilution). Bands were visualized with Immobilon Western Chemiluminescent HRP Substrate and analyzed using a FluorChemQ image analyzer to determine the protein expression levels.

2.7. Quantitative PCR

Total RNA was extracted from rat livers with the nucleic acid purification kit MagExtractor-RNA- (Toyobo, Osaka, Japan), and subsequently reverse transcribed with SuperScript VILO cDNA synthesis kit (Life Technologies) according to the manufacturer's instructions. The resultant cDNA was used as the template for quantitative PCR analyses with gene-specific primers (Life Technologies). The sequences of the primers were as follows: *Calr* (CRT gene), 5′-GAA GGA CAT GCA TGG AGA CTC AG-3′ (forward) and 5′-GTG TGA ATT CAT CAT CCT TAC ACC G-3′ (reverse); *Canx* (CNX gene), 5′-TGA CTG TAG CGT TGC CAG TG-3′ (forward) and 5′-CAC TCT TCT GTT TCT CTT CAA GC-3′ (reverse); and *B2m* (β2-microglobulin gene) as a housekeeping gene for normalization, 5′-CGA GAC CGA TGT ATA TGC TTG C-3′ (forward) and 5′-GTC CAG ATG ATT CAG AGC TCC A-3′ (reverse). Real-time PCR reactions were performed with a CFX96 real-time system (Bio-Rad, Hercules, CA, USA) in the presence of 400 nM for each primer, 10 ng of cDNA, and 10 μL of FastStart universal SYBR Green Master Mix (ROX) (Roche; Basel, Switzerland) for a total volume of 20 μL. The following PCR conditions were used for all samples: 95 °C for 10 min, and then 40 cycles of 95 °C for 5 s and 60 °C for 1 min. The fluorescence intensity was monitored at the end of each amplification step. At the end of the reactions, the melting curves and quantities were analyzed with a CFX manager application (Bio-Rad).

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