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Decreased expression of endoplasmic reticulum chaperone GRP78 in liver of diabetic mice

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ABSTRACT

To identify molecular targets associated with the development of diabetes, we analyzed the hepatic proteome of obese diabetic *db/db* mice using electrophoresis on a high-resolution two-dimensional gel combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. By comparison between non-diabetic *db*/+ and diabetic *db/db* mice, six proteins and one protein were significantly decreased and increased in the diabetic mice, respectively. Among these proteins, two of the decreased proteins are involved in endoplasmic reticulum (ER) stress-related unfolded protein response, GRP78 and protein disulfide isomerase A3, and it was revealed that the decreased GRP78 expression in the liver of diabetic *db/db* mice is due to the reduction of GRP78 protein synthesis rather than RNA transcription. In addition, we found that the treatment of human hepatocyte HepG2 cells with oleic acid decreased the expression of GRP78, and attenuated the activation of AKT by insulin stimulation. These results suggest that decreased GRP78 expression may induce resistance to insulin by inhibiting the AKT activation, and plays an important role in the development of type 2 diabetes.

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1. Introduction

Type 2 diabetes is one of the most prevalent and serious metabolic diseases globally, and peripheral insulin resistance and pancreatic β-cell dysfunction are the hallmarks of the disease [1,2]. Normal pancreatic β-cells can compensate for insulin resistance by increasing insulin secretion, but insufficient compensation leads to the onset of glucose intolerance. Once hyperglycemia becomes apparent, the insulin resistance and dysfunction of β-cells progressively worsen. Insulin sensitizers are partially effective at improving glucose disposal in skeletal muscle and suppressing hepatic gluconeogenesis, and prevent the progression of diabetes. However, the pathophysiology of the insulin-resistant state is still uncertain, and more detailed knowledge of the mechanism that leads to insulin resistance is necessary to identify new targets for the development of anti-diabetic drugs.

The endoplasmic reticulum (ER) is a large membrane-enclosed cellular compartment in which secretory and membrane-bound proteins are synthesized and folded into their final three-dimensional structures [3]. Protein folding in ER lumen is facilitated by a number of molecular chaperones and folding enzymes, including glucose-regulated protein (GRP) 94, GRP78 and protein disulfide isomerase (PDI) [4]. In stressful conditions such as the accumulation of misfolded proteins, however, the capacity of these proteins becomes inadequate, and leads to a condition defined as ER stress. The cellular response to ER stress, referred to as unfolded protein response (UPR), results in activation of three linked signaling pathways emanating from three ER stress sensors: inositol-requiring protein 1α (IRE1α), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6α (ATF6α) [5,6]. The combined actions of these signaling cascades serve to reduce ER stress through induction of chaperone and attenuation of protein translation. Previous studies have shown that ER stress triggers reduced insulin action in adipocytes and hepatocytes [7–10]. In addition, ER stress may account for altered insulin secretion from pancreatic β-cells [11,12], and defect of UPR may play a dominant role in the development of type 2 diabetes.

Here we analyzed the hepatic proteome of obese diabetic *db/db* mice using electrophoresis on a high-resolution two-dimensional gel combined with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and found that levels of two UPR-related proteins, GRP78 and PDI A3, were significantly decreased in the diabetic mice. In addition, we found that the treatment with oleic acid (OA) of human HepG2 cells decreased the expression of GRP78 and attenuated the activation of AKT, a serine/threonine kinase that regulates most of the metabolic actions of insulin. The significance of the altered expression of GRP78 during the development of diabetes is discussed.

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2. Materials and methods

2.1. Animals and preparation of hepatocytes

Male C57BLKS/J $m^{+}/Lepr^{db}$ (*db*/+) and C57BLKS/J $Lepr^{db}/Lepr^{db}$ (*db*/*db*) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed with a 12-h light/dark cycle (lights off: 20:00–08:00) and ambient temperature regulated at 22 ± 2 °C. All mice were fed standard rodent chow and tap water ad libitum. Non-fasting blood glucose levels were determined weekly by the glucose oxidase method [13]. Data on body weights and plasma glucose levels over 14 weeks are summarized in Fig. 1A. For preparation of hepatocytes, male *db*/+ and *db*/*db* mice were anesthetized with pentobarbital, and hepatocytes were isolated after collagenase perfusion of liver and cultured following the published procedures with minor modification [14]. All animal studies were performed in accordance with the protocols approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

2.2. Proteomic analysis

Preparation of protein extracts and proteomic analysis were performed as described previously [15]. Briefly, each protein extract (500 μg of protein) was loaded onto dry immobilized pH gradient strips (17 cm, pH 3–10 non-linear, Bio-Rad Laboratories, Hercules, CA, USA). Isoelectric focusing was performed at a maximum of 10,000 V for a total of 80,000 V h. Two-dimensional electrophoresis was performed in 12% SDS–polyacrylamide gels, and the gels were stained with a SYPRO Ruby solution (Bio-Rad Laboratories). Differentially expressed spots were defined as those that had a difference in expression levels between control and diabetic mice of more than 2-fold on the basis of Student's *t*-test. The spots were excised and digested with Trypsin, and the resultant peptides were extracted and analyzed using a Voyager-DE™ MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Proteins were identified with peptide mass fingerprinting using an MS-Fit search engine.

2.3. Western blot analysis

Equal amounts of protein samples (20 μg each) were separated on 10% SDS–polyacrylamide gels and blotted onto a nitrocellulose membrane by electrotransfer. Immunodetection was performed with a chemiluminescent detection method (ECL plus, GE Healthcare Bioscience, Piscataway, NJ, USA). Antibodies used in this study were as follows: anti-GRP78 (Pharmingen), anti-phospho-IRE1α (NB100-2323, Novus Biologicals, Littleton, CO, USA), anti-ATF6α (sc-22799, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-α-tubulin (#T9026, Sigma Chemical, St. Louis, MO, USA), anti-phospho-eIF2α (#3597, Cell Signaling Technology, Danvers, MA, USA), anti-eIF2α (#9722, Cell Signaling Technology), anti-phospho-AKT (Ser473) (#9271, Cell Signaling Technology) and anti-AKT (#9272, Cell Signaling Technology).

2.4. RNA isolation and gene expression

Total RNA from mouse liver was isolated using GenElute mammalian total RNA miniprep kit (Sigma Chemical). The RNA was reverse-transcribed to single-strand cDNA using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) and the resultant cDNA was used for real-time PCR analysis using Realtime PCR master mix (TOYOBO) and TaqMan probe for mouse GRP78. Data were normalized to 18S rRNA and are presented as means ± SD of three independent experiments for each sample ($n = 5$).

2.5. ^{35}S-Methionine labeling and immunoprecipitation

Mouse hepatocytes were seeded in a 6-well plate (1×10^6 cells/well) at 24 h before the experiment. The following day, the cells were washed once with phosphate-buffered saline (PBS) and incubated in fresh medium without methionine and cysteine at 37 °C for 1 h, and then incubated in the medium containing 100 mCi/ml ^{35}S-Methionine (EasyTag EXPRE^{35}S^{35}S Protein Labeling Mix, Perkin Elmer, Waltham, MA, USA) for 1–24 h at 37 °C. At indicated time points, the cells were washed twice with PBS and lysed in ice-cold lysis buffer (10 mM Tris–HCl, pH7.5, 1% NP-40, 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The lysates were then centrifuged at 20,000*g* for 10 min at 4 °C, and the aliquot of supernatant (100 μg of protein) was used for immunoprecipitation using anti-GRP78 (sc-13968, Santa Cruz Biotechnology) or control anti-rabbit IgG antibody. The immunoprecipitated proteins were resolved by SDS–PAGE, and the dry gel was exposed to X-ray film (Fuji Film, Kanagawa, Japan) at −80 °C for 24–72 h.

2.6. Cell culture and treatment with fatty acid

OA and palmitic acid (PA) were conjugated to FA-free bovine serum albumin (BSA) by dissolving them and mixing with an aqueous BSA solution until homogeneous. HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum with 95% air and 5% CO_2. For the treatment with fatty acid, cells were grown in the medium with 0.2 mM OA–BSA conjugates, 0.2 mM PA–BSA conjugates or BSA for the indicated period.

2.7. Oil Red O staining

HepG2 cells were incubated in the medium with 0.2 mM OA–BSA conjugates, 0.2 mM PA–BSA conjugates or BSA for 96 h. Cells were washed with PBS, fixed for 1 h with 4% paraformaldehyde in PBS and then immersed in 60% isopropanol for less than 1 min. Then, cells were stained with 1% Oil Red O in 60% isopropanol for 1 h at room temperature.

3. Results

3.1. Proteomic analysis of liver of diabetic db/db mice

To identify the proteins associated with the development of diabetes, we compared the expression profile of proteins in the liver of non-diabetic *db*/+ mice and diabetic *db*/*db* mice using 2-DE. Fig. 1B shows typical images of the gels. About 700 spots in each image were matched and quantified using PDQuest analyzing software, and six proteins and one protein were found to be expressed at decreased levels and in increased levels in diabetic *db*/*db* mice compared with those in non-diabetic *db*/+ mice, respectively (Fig. 1C). To identify these proteins, the protein spots were excised from the 2-D gels and analyzed by peptide mass fingerprinting after in-gel digestion with trypsin. The results are summarized in Supplementary Table 1. Among these proteins, the decreased expressions of GRP78, Hsc70, PDIA3 and GSTP1 were confirmed by Western blotting using the commercially available antibodies in liver of diabetic *db*/*db* mice (Fig. 2).

3.2. Decreased GRP78 expression in the liver of db/db mice is due to reduction of GRP78 protein synthesis rather than RNA transcription

A previous study demonstrated that mice with genetic (*ob*/*ob* mice) or diet-induced obesity show an elevation of several biochemical indicators for ER stress and GRP78 mRNA in the liver

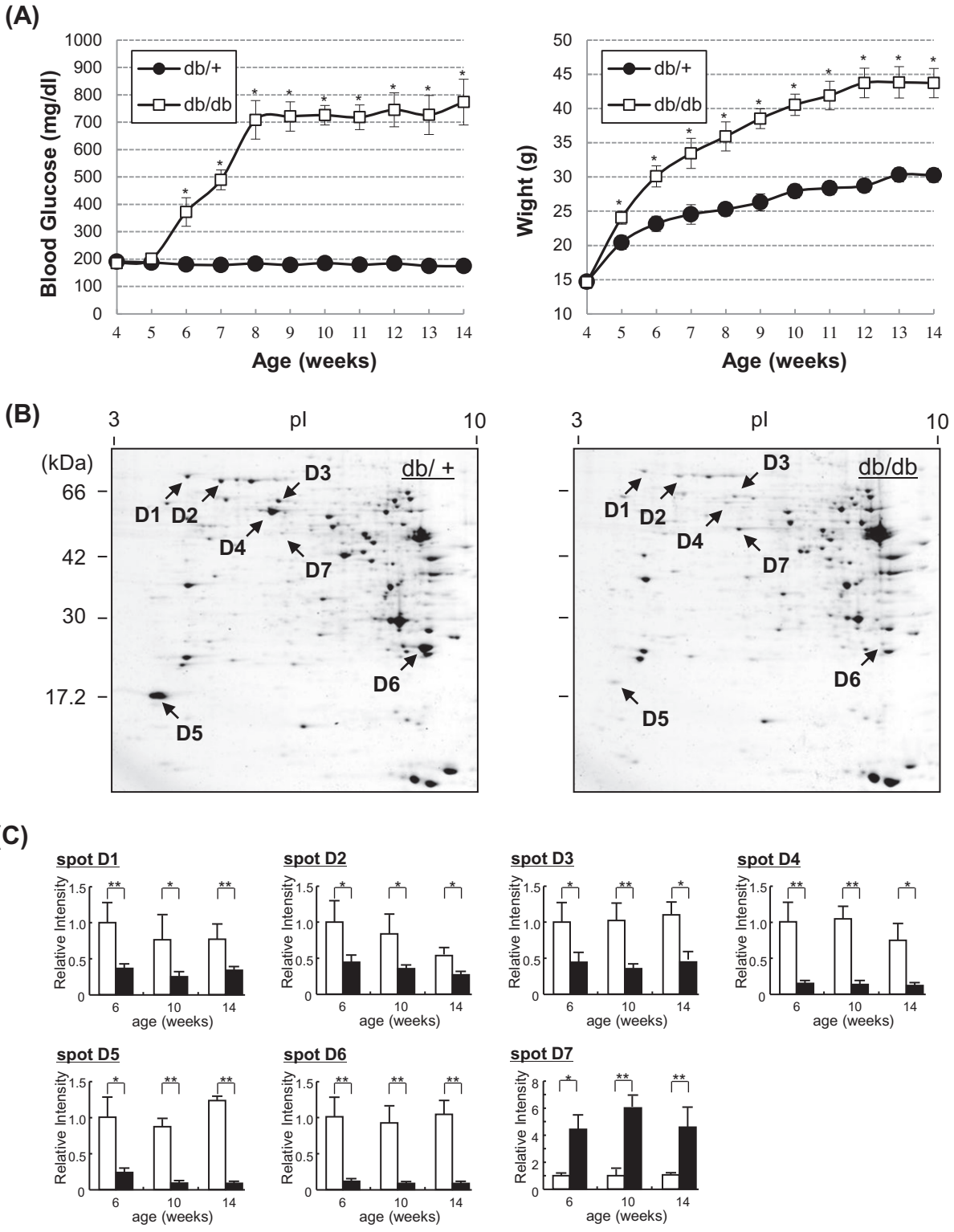

Fig. 1. Comparison of proteins expressed in the liver of non-diabetic *db*/+ mice and diabetic *db*/*db* mice by 2-DE. (A) Changes of body weight (left panel) and plasma blood glucose (right panel) in *db*/+ mice and *db*/*db* mice. Blood glucose levels were determined by the glucose oxidase method. (B) Protein extracts (500 μg of proteins) from liver of *db*/+ mice and *db*/*db* mice at 10 weeks of age were separated by 2-DE and stained with SYPRO Ruby solution. Arrows indicate the spots expressed differently between *db*/+ mice and *db*/*db* mice. (C) The relative intensity of the spots D1–D7 in liver of *db*/+ mice and *db*/*db* mice at 6, 10 and 14 weeks of age was quantified with PDQuest analyzing software.

and adipocytes [7]. However, our results showed that the expression of UPR-related proteins, GRP78 and PDI A3, was decreased in the liver of diabetic *db*/*db* mice compared with that of non-diabetic *db*/+ mice. To evaluate whether ER stress is increased in the liver of diabetic *db*/*db* mice, we next examined the expression of the activated forms of ER stress sensor proteins, such as IRE1α and ATF6α. As shown in Fig. 3A, phosphorylated IRE1α was detected in the liver of diabetic *db*/*db* mice but not non-diabetic

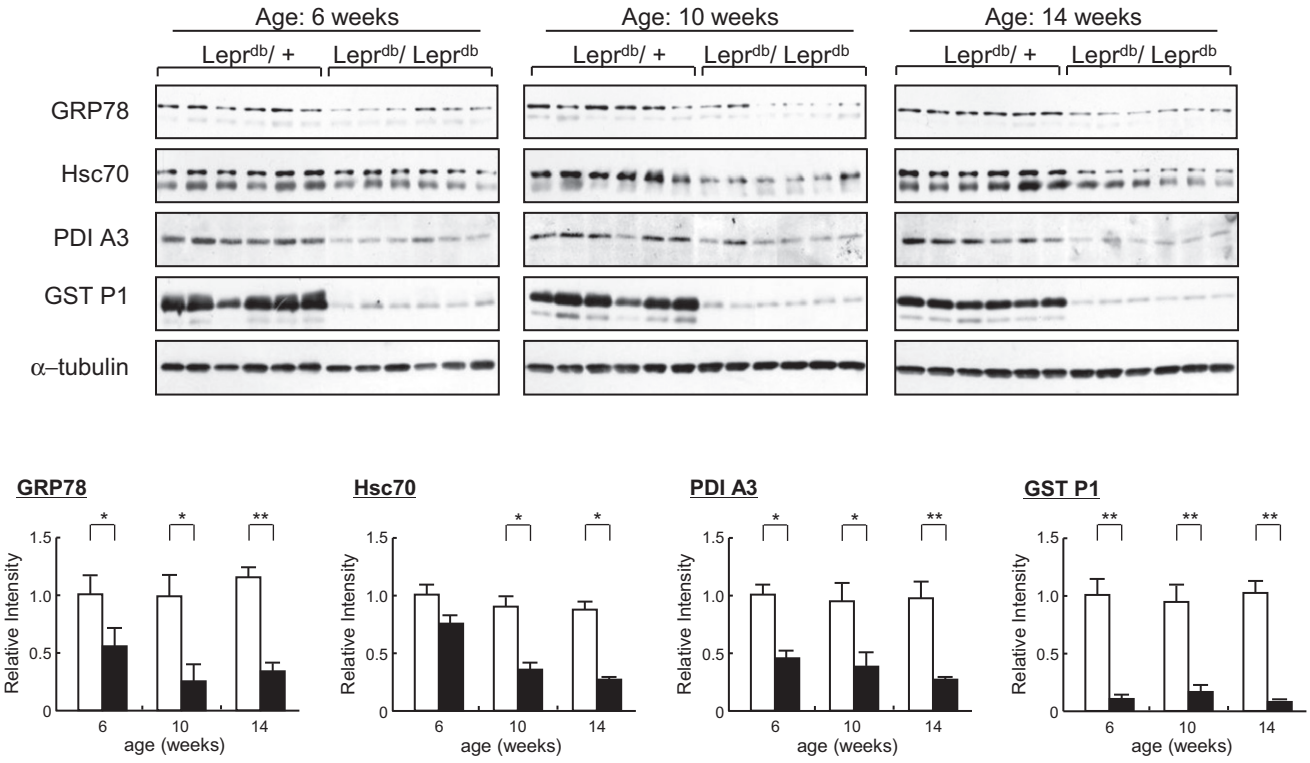

Fig. 2. Decreased expression of GRP78, Hsc70, PDIA3 and GSTP1 in the liver of diabetic db/db mice. Protein extracts (20 μg each) from liver of *db*/+ mice and *db*/*db* mice at 6, 10 and 14 weeks of age were separated by 12% SDS–PAGE, and expression levels of GRP78, Hsc70, PDIA3 and GSTP1 were analyzed by Western blotting using the respective antibodies. The intensity of the bands was quantified using Quantity One Image analysis software (BioRad Laboratories). The values are the mean ± SD from each group ($n = 6$). Statistical significance was determined using unpaired Student's *t*-test. $^{*}P < 0.01$, $^{**}P < 0.001$. White bars, non-diabetic *db*/+ mice; black bars, diabetic *db*/*db* mice.

db/+ mice at 4–14 weeks of age. Furthermore, activation of ATF6α, detected as the processed form of ATF6α, was also observed in the liver of diabetic *db*/*db* mice but not non-diabetic *db*/+ mice. In contrast, the level of phosphorylated eIF2α, which is phosphorylated by PERK, decreased in the liver of diabetic *db*/*db* mice compared with that of non-diabetic *db*/+ mice, suggesting that the PERK pathway did not respond to ER stress in the diabetic *db*/*db* mice. Thus, ER stress is actually increased, but the UPR for ER stress may be defective in the diabetic *db*/*db* mice.

Next, we examined the expression level of GRP78 mRNA. As shown in Fig. 3B, the level of GRP78 mRNA in the liver of *db*/*db* mice compared with that of *db*/+ mice was somewhat increased at 6 weeks of age, and then decreased at 10 or 14 weeks of age. In contrast, the level of GRP78 protein was not different in the liver of *db*/*db* mice and *db*/+ mice at 4 weeks of age, but was significantly decreased at 5 weeks of age in the liver of *db*/*db* mice compared with that of *db*/+ mice (Fig. 3A). In addition, when the synthesis of GRP78 was measured by the labeling of cells with ^{35}S-methionine and immunoprecipitation using anti-GRP78 antibody, we observed that the synthesis of GRP78 was reduced in the hepatocytes derived from 6-week-old *db*/*db* mice compared with those from *db*/+ mice (Fig. 3C). Thus, the decreased GRP78 expression in the liver of diabetic *db*/*db* mice seems to be due to the reduction of GRP78 protein synthesis rather than RNA transcription.

3.3. Decreased GRP78 expression and insulin resistance in HepG2 cells treated with OA but not PA

Prolonged high glucose exposure has been proposed to induce ER stress [16–18], and ER stress and activation of UPR signaling pathways play a dominant role in the development of type 2 diabetes [7–10]. However, we showed that the decreased expression of GRP78 in the liver of diabetic *db*/*db* mice preceded the elevation of blood glucose (Figs. 1A and 3A). In contrast, lipid accumulation in the liver of diabetic *db*/*db* mice was observed at 5 weeks of age (data not shown). As chronic elevation in plasma free fatty acid levels is commonly associated with impaired insulin-mediated glucose uptake [19,20], we next examined whether the accumulation of lipid triggers the decreased GRP78 expression. When human hepatocyte HepG2 cells were cultured in the medium containing OA or PA, it was shown that a large number of lipid droplets were accumulated in the cells by Oil Red O staining (Fig. 4A), and GRP78 expression was drastically reduced by the treatment with OA, but was increased in the cells treated with PA (Fig. 4B). Furthermore, the insulin-mediated phosphorylation of AKT at Ser 473 was diminished in the cells treated with OA but not PA (Fig. 4C). Thus, the decrease of GRP78 expression seemed to be accompanied by insulin resistance, such as decreased phosphorylation of AKT.

4. Discussion

To identify the proteins associated with the development of diabetes, we conducted a proteomic analysis in the liver of *db*/*db* mice and found that levels of two UPR-related proteins, GRP78 and PDI A3, were significantly decreased in the diabetic mice. GRP78 is a member of the HSP70 family abundant in the lumen of ER, and plays important roles as a molecular chaperone that removes malfolded proteins in ER lumen. Overexpression of GRP78 has been shown to increase ER stress resistance and to have beneficial effects in several cell types [21–23]. Therefore, the decreased expression of GRP78 in the diabetic mice may impair 'quality control' in the ER, resulting in many abnormal changes in various tissues in the form of long-term complications like diabetic retinopathy and nephropathy.

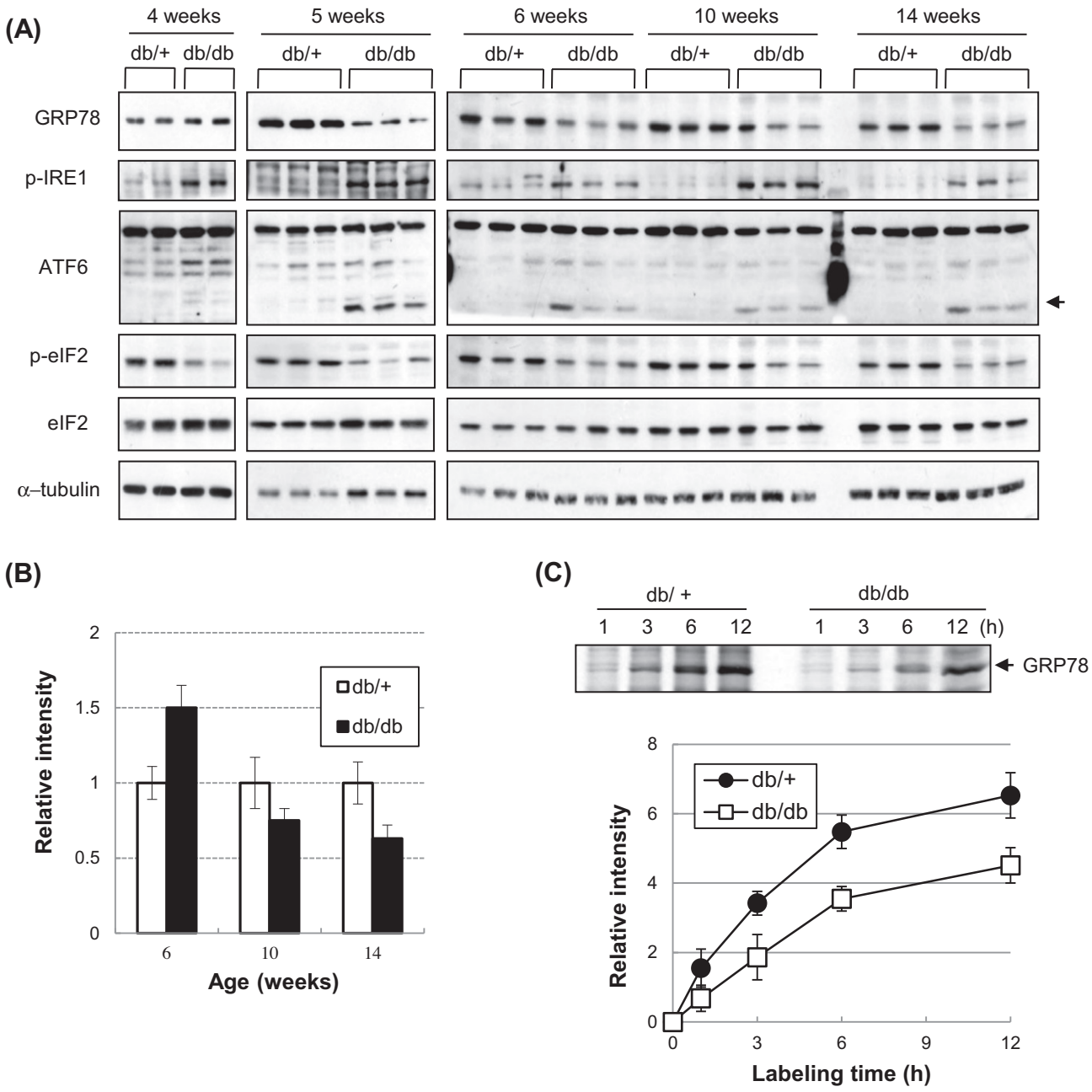

Fig. 3. Decreased GRP78 expression is due to the reduced steady-state levels of GRP78 protein rather than its transcriptional level in the liver of db/db mice. (A) Protein extracts (20 μg each) from the liver of control *db*/+ mice and diabetic *db*/*db* mice were separated by 10% SDS–PAGE, and expression levels of GRP78, phospho-IRE1α, ATF6α and α-tubulin were analyzed by Western blotting using the respective antibodies. (B) Total RNA (0.5 μg each) from liver of non-diabetic *db*/+ mice and diabetic *db*/*db* mice was reverse-transcribed to single-stranded cDNA and analyzed by PCR using primers specific for *grp78* mRNA. Raw expression values of *grp78* mRNA were normalized to 18S rRNA. The values are the mean ± SD from each group ($n = 6$). Statistical significance was determined using unpaired Student's *t*-test. $^{*}P < 0.01$. White bars, non-diabetic *db*/+ mice; black bars, diabetic *db*/*db* mice. (C) Hepatocytes derived from 6-week-old *db*/*db* mice or *db*/+ mice were labeled with ^{35}S-methionine for the indicated times. Then, the cells were lysed and GRP78 was immunoprecipitated using anti-GRP78 antibody. The immunoprecipitates were resolved with SDS–PAGE and GRP78 was detected by autoradiography. The values are the mean ± SD from three independent experiments. Statistical significance was determined using unpaired Student's *t*-test. $^{*}P < 0.05$.

As for the expression levels of GRP78 in diabetes, Parfett et al. reported that transient increased expression of hepatic GRP78 mRNA in spontaneously diabetic NOD mice coincides with the transition from the prediabetic to the diabetic stage in which there is measurable hyperglycemia [24]. In contrast, the expression of GRP78 protein significantly decreased in the liver of spontaneously diabetic Zucker rats at 12 weeks of age, but no significant change was detected in its mRNA level compared with that of normal rats [25]. Here, we revealed that GRP78 mRNA increased significantly in the liver of diabetic *db*/*db* mice at 6 weeks of age, probably due to ER stress and UPR stimulation, but decreased thereafter even under conditions of prolonged ER stress. In contrast, GRP78 protein was significantly decreased in the liver of *db*/*db* mice even at 5 weeks of age. Thus, the decreased GRP78 expression in the liver of diabetic *db*/*db* mice seems to be due to the reduction of GRP78 protein synthesis rather than RNA transcription.

A previous report by Ozcan et al. [7] sheds more light on the links between obesity, ER stress, insulin action and type 2 diabetes. They showed that obesity causes a considerable elevation of ER stress, and this in turn plays a central role in the development of

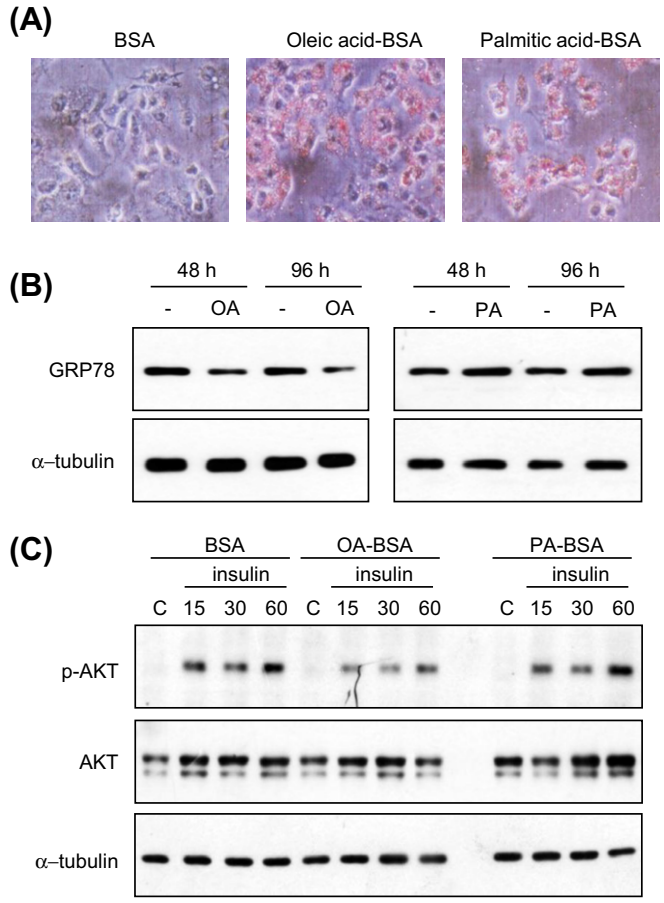

Fig. 4. Effect of fatty acid on GRP78 expression and AKT phosphorylation in HepG2 cells. (A) Light micrographs of HepG2 cells incubated in the medium with 0.2 mM OA–BSA conjugates, 0.2 mM PA–BSA conjugates or BSA for 96 h. (B) HepG2 cells were incubated in the medium with 0.2 mM OA–BSA conjugates, 0.2 mM PA–BSA conjugates or BSA for 96 h. Then, the cells were harvested and analyzed for expression of GRP78 and α-tubulin by Western blotting using their respective antibodies. (C) HepG2 cells were incubated in the medium with 0.2 mM OA–BSA conjugates, 0.2 mM PA–BSA conjugates or BSA for 96 h. Then, the cells were treated with insulin for the indicated period before cell harvesting. Western blot analysis was performed with phosphor-AKT and total AKT antibodies.

insulin resistance and diabetes by triggering c-Jun N-terminal kinase (JNK) activity via IRE1α and inhibition of insulin receptor signaling. They also observed an elevation of GRP78 mRNA in the liver and adipocytes. In the present study, we confirmed the elevation of GRP78 mRNA in the liver of diabetic *db/db* mice at 6 weeks of age but not thereafter. In contrast, the expression level of GRP78 protein was decreased in the liver of diabetic *db/db* mice even at 5 weeks of age and thereafter. GRP78 is not only involved in protein folding in ER lumen as a molecular chaperone, but also negatively regulates ER stress sensors such as IRE1α, PERK and ATF6α [26]. Therefore, decreased expression of GRP78 protein in ER may contribute to prolonged ER stress response.

Prolonged high glucose exposure has been proposed to induce ER stress [16–18], and the activation of UPR signaling pathways was suggested to play a dominant role in the development of type 2 diabetes [7–10]. However, we showed that hyperglycemia was preceded by decreased GRP78 protein level in the liver of diabetic *db/db* mice, suggesting that the decreased expression of GRP78 is not a result of hyperglycemia in diabetic *db/db* mice. In contrast, lipid accumulation in the liver of diabetic *db/db* mice was observed at 5 weeks of age. Additionally, we showed that GRP78 expression was decreased by the treatment with OA but not PA in human hepatocyte HepG2 cells. Recent studies have shown that saturated fatty acids, such as PA, induce ER stress and cell death in pancreatic β-cells and hepatocytes, whereas unsaturated fatty acids, such as OA, do not [27,28]. Further study is needed to clarify the mechanism by which oleic acid causes the reduction of GRP78 expression.

A recent report has shown that targeted knockout of GRP78 suppresses the activation of AKT, a serine/threonine kinase that regulates growth factor signaling, and blocks tumorigenesis of Pten-null prostate epithelium cancer [29]. Activation of AKT also regulates most of the metabolic actions of insulin, such as the suppression of hepatic glucose production, the regulation of glycogen synthesis in liver and the activation of glucose transport in muscle and adipocytes [30], and AKT activity decreases in insulin-resistant tissues of aging and obese mice [31,32]. Thus, the decreased expression level of GRP78 may be associated with the decreased activation of AKT and the development of insulin resistance.

In summary, we revealed here the decreased expression of GRP78 protein in the liver of diabetic *db/db* mice. The treatment of hepatocytes with oleic acid decreased the expression of GRP78 and attenuated the activation of AKT by insulin stimulation. The decreased GRP78 expression in the diabetic mice may be associated with the induced resistance to insulin by inhibiting the AKT activation. These findings may provide clues for the development of effective clinical treatments for diabetes and its complications.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.118.

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