Endoplasmic Reticulum Stress Induces the Expression of Fetuin-A to Develop Insulin Resistance

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Fetuin-A is a biomarker reported to be important in many metabolic disorders, including obesity, diabetes, and hepatic steatosis. Although it is well known that fetuin-A is increased in diabetes and nonalcoholic fatty liver disease (NAFLD), the levels of fetuin-A in diabetic patients with NAFLD are unknown. Furthermore, the regulation of fetuin-A expression is still obscure. In this study, a total of 180 age- and sex-matched subjects with normal glucose tolerance, NAFLD, newly diagnosed diabetes (NDD), and NDD with NAFLD were recruited. We found that the levels of fetuin-A were significantly increased in NDD with NAFLD as compared with NDD or NAFLD subjects. We further used HepG2 cells to investigate the regulation of fetuin-A. Treatment with endoplasmic reticulum (ER) stress activator, thapsigargin, increased the expression of fetuin-A mRNA and protein in a time- and dose-dependent manner. Pretreatment with ER stress inhibitor, 4-phenylbutyrate, reversed high glucose or palmitate-induced fetuin-A expression. Moreover, treatment with 4-phenylbutyrate in both streptozotocin-induced and high-fat diet-induced diabetic mice not only decreased hepatic fetuin-A levels but also improved hyperglycemia. Taken together, we found that fetuin-A levels were increased in diabetes patients with NAFLD. Moreover, ER stress induced by high glucose and palmitate increased the expression of fetuin-A and further contributed to the development of insulin resistance. (***Endocrinology*** **153: 2974–2984, 2012)**

Fetuin-A (α-2-Heremans Schmid glycoprotein), a 64-kDa glycoprotein produced in the liver and secreted into circulation (1), is an important circulating inhibitor of vascular calcification *in vivo* (2). Previous studies find that in patients with end-stage renal disease, serum fetuin-A concentrations were significantly lower and independently associated with risk of cardiovascular and all-cause mortality (3, 4). However, serum fetuin-A is also associated with subclinical inflammation and positively correlated with C-reactive protein (CRP) levels in subjects with coronary artery disease and the general population (5, 6). Moreover, many cohort studies report that fetuin-A levels are significantly associated with obesity, hepatic steatosis (5, 7, 8), diabetes (9), and the risk of diabetes (6), myocardial infarction, and ischemic stroke (10). Although fetuin-A acts as a biomarker for diabetes and hepatic steatosis, the levels of fetuin-A in diabetic patients with hepatic steatosis are not clear. Previous studies have demonstrated that nonalcoholic fatty liver disease (NAFLD) is highly prevalent among patients with type 2 diabetes ($\approx$70%) (11), and the concurrence of NAFLD and diabetes leads to a poorer outcome independent of traditional cardiovascular risk factors (12–14).

Fetuin-A is an important promoter of insulin resistance (15–22). Elevated levels of fetuin-A are observed in db/db

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Abbreviations: A1C, Hemoglobin A1c; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CHOP, C/EBP homologous protein; CRP, C-reactive protein; CV, coefficient of variation; eGFR, estimated glomerular filtration rate; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; GTT, glucose tolerance test; HDL, high-density lipoprotein; HFD, high-fat diet; HOMA-IR, homeostasis model assessment-insulin resistance; IPGTT, ip GTT; ITT, insulin tolerance test; JNK, c-Jun N-terminal kinase; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NDD, newly diagnosed diabetes; NGT, normal glucose tolerance; 4-PBA, 4-phenylbutyrate; PERK, protein kinase R-like ER kinase; STZ, streptozotocin; TPG, thapsigargin.

mice and high-fat diet (HFD)-induced insulin-resistant rats (18). Treatment of wild-type mice with fetuin-A induces insulin resistance (19), whereas fetuin-A knockout mice have improved insulin sensitivity and are resistant to HFD-induced weight gain (20, 21). It is known that fetuin-A induces insulin resistance by inhibition of the insulin receptor tyrosine kinase activity in skeletal muscle and hepatocytes (15–17). However, the factors that mediate the expression of fetuin-A are still unclear. Recent studies report that palmitate stimulates nuclear factor κB binding to the fetuin-A promoter to increase the expression of fetuin-A (18), and high glucose enhances fetuin-A expression through activation of ERK1/2 (22).

Both hyperlipidemia and hyperglycemia induce endoplasmic reticulum (ER) stress and promote the activation of unfolded protein response (23–28), which are associated with the pathogenesis of many diseases, such as diabetes, cardiovascular disease, and neurodegenerative disorders (29). In macrophages, cholesterol-loading activates unfolded protein response in the ER (23). Furthermore, animal studies show that hyperglycemia is associated with ER stress in the liver of streptozotocin (STZ)-induced diabetic mice (24) and that infusion of glucose and lipids in nondiabetic rats increases ER stress and causes insulin resistance in the liver (25). The increased ER stress also causes insulin resistance in both human and rat myotubes and impairs glucose transporter 4 (GLUT4) production and insulin-induced glucose uptake (26). In addition to its effect on insulin sensitivity, elevated ER stress increases apoptosis in cultured insulinoma MIN6 cells and may be responsible for the loss of β-cell mass in diabetic mice (27). Therefore, treatment of type 2 diabetic mice with ER stress inhibitors results in normalization of hyperglycemia, restoration of systemic insulin sensitivity, resolution of fatty liver disease, and enhancement of insulin action in liver, muscle, and adipose tissues (28). Although it is well known that elevated ER stress is associated with the pathogenesis of diabetes, the relationship between ER stress and fetuin-A is still unknown.

This study not only investigates the levels of fetuin-A in subjects with diabetes and NAFLD, either alone or concurrent, but also evaluates the effects of ER stress inhibitors on the hepatic expression of fetuin-A in STZ-induced insulin-deficient diabetic mice and in HFD-induced insulin-resistant diabetic mice. It also clarifies the possible mechanism that regulates the expression of fetuin-A.

Materials and Methods

Human subjects

The study protocol was approved by the Human Experiment and Ethics Committee of National Cheng Kung University Medical Center, and all eligible subjects gave written informed consent before participation. From June 2007 to July 2008, all subjects who had been admitted for a physical checkup at the Preventive Health Center of National Cheng Kung University Hospital were screened. All healthy subjects who did not have a medical history of diabetes received a standard 75-g oral glucose tolerance test (GTT) after a 10-h overnight fast, a normal diet for 3 d before the test, and abstention from smoking for more than 24 h. None of the women were pregnant when tested. Each subject was assessed by abdominal ultrasound to diagnose the presence or absence of NAFLD. Diabetes and normal glucose tolerance (NGT) were defined according to American Diabetes Association criteria: NGT, if fasting plasma glucose was less than 100 mg/dl and 2-h postload glucose was less than 140 mg/dl without a history of diabetes; diabetes, if fasting plasma glucose was more than or equal to 126 mg/dl or 2-h postload glucose more than or equal to 200 mg/dl. None of the diabetic patients had been diagnosed as having diabetes or had been treated with insulin or an antidiabetic agent before. Each consecutive index diabetic subject was then matched to the first subject of the same gender in the other three groups from the list who had the same age. If an exact age match could not be found, then the first subject closest to the age of the index subject (within ±1 yr) was picked. Using this method, we were able to select 180 age- and sex-matched subjects of NGT (n = 45), newly diagnosed diabetes (NDD) (n = 45), NGT with NAFLD (NAFLD, n = 45), and NDD + NAFLD (n = 45) groups.

After an overnight 12-h fast, all subjects received a blood test, including routine biochemistry, fasting plasma glucose, hemoglobin A1c (A1C), adiponectin, and fetuin-A. All subjects received a standard 75-g oral GTT. The estimated glomerular filtration rate (eGFR) (ml/min per 1.73 m^2) was calculated using the modification of diet in renal disease equation. Wearing light indoor clothes, each subject's body height (to the nearest 0.1 cm), weight (to the nearest 0.1 kg), and waist circumference (to the nearest 0.1 cm) were measured. Waist circumference measurement was performed at the end of normal expiration in duplicate on bare skin midway between the lower rib margin and the iliac crest. Body mass index (BMI) (in kg/m^2) was calculated as weight (in kilograms) divided by height (in meters) squared. Central obesity was defined as waist circumference more than or equal to 90 cm in males and more than or equal to 80 cm in females. For blood pressure measurement, subjects were resting in a supine position in a quiet ambience, and measurements were obtained in a fasting state between 0800 and 1000 h. Two blood pressure readings, separated by intervals of at least 5 min, were taken with an appropriate-sized cuff wrapped around the right upper arm using a DINAMAP vital sign monitor (model 1846SX; Critikon, Inc., Irvine, CA). Subjects with a systolic blood pressure more than or equal to 140 mm Hg or diastolic blood pressure more than or equal to 90 mm Hg were defined as having hypertension. Liver ultrasound was performed by an experienced radiologist with high-resolution ultrasonography (Xario SSA-660A; Toshiba, Nasu, Japan) using a 3.5-MHz linear transducer. The NAFLD diagnostic criteria included characteristic echo patterns of hepatorenal echo contrast, bright liver, deep (posterior beam) attenuation, and vascular blurring.

Blood glucose was measured by a hexokinase method (Roche Diagnostic GmbH, Mannheim, Germany). Serum insulin (Mercodia AB, Uppsala, Sweden) was measured by ELISA. Insulin resistance was defined by the homeostasis model assessment-

TABLE 1. Clinical characteristics among NGT subjects and NDD subjects with or without NAFLD

	NGT	NDD	NAFLD	NDD + NAFLD	*P*
n	45	45	45	45	
Age (years)	61 ± 11	61 ± 11	61 ± 10	60 ± 11	NS
Sex (F/M)	20/25	20/25	20/25	20/25	NS
Body weight (kg)	58.8 ± 10.7	61.2 ± 10.2	67.7 ± 10.6[b,c]	71.8 ± 12.7[b,d,f]	<0.001
Waist circumference (cm)	79.1 ± 8.7	83.0 ± 9.4	88.9 ± 9.3[b,c]	93.8 ± 8.4[a,d,f]	<0.001
BMI (kg/m^2)	22.4 ± 2.4	23.2 ± 3.2	25.8 ± 3.0[b,c]	27.9 ± 3.0[a,d,f]	<0.001
Systolic blood pressure (mm Hg)	123 ± 17	132 ± 18[a]	128 ± 18[b]	129 ± 17[b]	<0.05
Diastolic blood pressure (mm Hg)	71 ± 11	77 ± 10[b]	76 ± 12[b]	79 ± 10[a]	<0.01
Fasting plasma glucose (mg/dl)	84 ± 7	135 ± 55[a]	91 ± 6[d]	149 ± 58[a,e,g]	<0.01
Postload 2 h glucose (mg/dl)	95 ± 24	258 ± 92[a]	109 ± 20[b,e]	267 ± 110[a,g]	<0.001
A1C (%)	5.6 ± 0.3	7.3 ± 2.0[b]	5.8 ± 0.3[d]	8.0 ± 2.2[a,c,g]	<0.001
ALT (U/liter)	24 ± 6	29 ± 17	29 ± 11	32 ± 18	NS
AST (U/liter)	21 ± 9	30 ± 25	31 ± 16	44 ± 43	NS
Creatinine (mg/dl)	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	NS
eGFR (ml/min per 1.73 m^2)	94.2 ± 19.3	92.2 ± 20.4	92.6 ± 17.8	90.6 ± 17.0	NS
CRP (mg/liter)	1.78 ± 3.24	3.69 ± 4.91[b]	3.86 ± 4.60[b]	5.09 ± 4.91[a,c,g]	<0.01
Total cholesterol (mg/dl)	199 ± 31	211 ± 40	209 ± 31	209 ± 38	NS
Triglyceride (mg/dl)*	90 ± 26	132 ± 69[b]	144 ± 67[b]	176 ± 86[a,d,g]	<0.001
HDL cholesterol (mg/dl)	59 ± 19	53 ± 14	53 ± 14	44 ± 10[b,g]	<0.001
LDL cholesterol (mg/dl)	122 ± 29	132 ± 34	133 ± 29	129 ± 33	NS
LDL/HDL-cholesterol ratio	2.3 ± 1.0	2.7 ± 0.8	2.9 ± 1.0[b]	3.1 ± 1.0[a]	<0.001
HOMA-IR	0.4 ± 0.3	1.0 ± 0.9[b]	1.0 ± 0.9[b]	2.9 ± 2.3[a,d,g]	<0.001
Adiponectin (μg/ml)	13.6 ± 7.6	9.6 ± 5.4[b]	9.6 ± 7.1[b]	7.6 ± 4.6[a,c,g]	<0.001

Data expressed as means ± SD. NS, Not significant. *Asterisk*, Kruskal-Wallis test. *P* values: ANOVA among groups. Student's *t* test: 1) compared with NGT: [b], $P < 0.01$; [a], $P < 0.001$; 2) compared with NDD: [c], $P < 0.05$; [d], $P < 0.01$; [e], $P < 0.001$; and 3) compared with NAFLD: [f], $P < 0.05$; [g], $P < 0.01$.

insulin resistance (HOMA-IR) index as [fasting insulin (μU/ml) × fasting plasma glucose (mM)]/22.5 (30). Serum total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol levels were determined in the central laboratory of National Cheng Kung University Medical Center with an autoanalyzer (Hitachi 747E; Hitachi, Tokyo, Japan). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. A1C was measured with a high performance liquid chromatographic method (Tosoh Automated Glycohemoglobin Analyzer HLC-723 GHbV A1c 2.2; intraassay coefficient of variation (CV) of 0.5%, interassay CV of 2.0%; Tokyo, Japan).

Serum fetuin-A was determined by an ELISA method (intraassay CV of 2.7%, interassay CV of 3.2%; Biovendor Laboratory Medicine, Brno, Czech Republic). The determination of serum adiponectin was carried out using AssayMax Human Adiponectin (Acrp30) ELISA kits (intraassay CV of 2.5%, interassay CV of 6.5%; AssayPro, St. Charles, MO). CRP was measured using a highly sensitive ELISA kit (Immunology Consultants Laboratory, Newberg, OR). Subjects with the following conditions or diseases were excluded: 1) alcohol consumption more than or equal to 20 g/d in the last year; 2) serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels more than two times the normal limit; 3) a positive test for hepatitis B surface antigen, hepatitis C antibody, and other causes of liver disease; 4) serum creatinine more than 1.5 mg/liter; 5) any acute or chronic inflammatory disease as determined by a leukocyte count more than 10,000/mm^3 or clinical signs of infection; and 6) any other major diseases, including generalized inflammation or advanced malignant diseases contraindicating this study.

Cell culture

HepG2 cell line (ATCC HB-8065) was a gift from Y. S. Tsai (Institute of Clinical Medicine, College of Medicine, National Cheng Kung University). A previous study indicated that fetuin-A is secreted from HepG2 cells (18). Therefore, HepG2 cells were used as a model to evaluate the possible mechanisms of fetuin-A expression. Cells were maintained (5% CO_2, 37 C) in DMEM (HyClone, South Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum. The cells were cultured in low-glucose DMEM without serum overnight and then harvested at the times indicated in the figures. The cells were pretreated with 25 μM PD98059, 25 μM SB203580, 25 μM

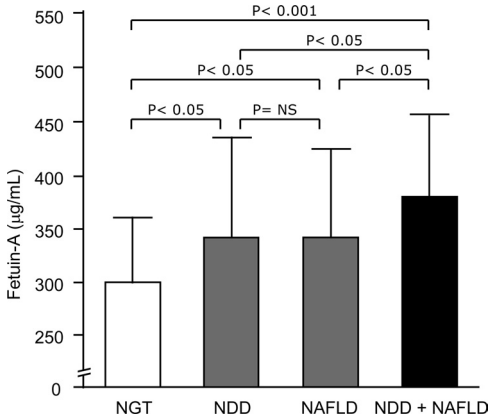

FIG. 1. Serum levels of fetuin-A in subjects with NGT, NDD without NAFLD (NDD), NGT with NAFLD (NAFLD), and NDD with NAFLD (NDD+NAFLD). $P < 0.001$ test for trend. Data are shown as mean ± SD. NS, Not significant.

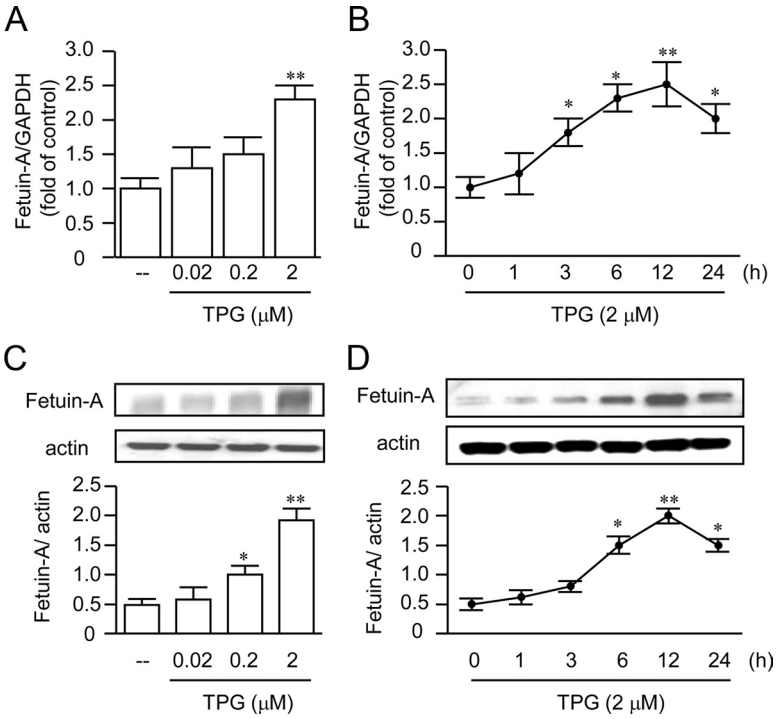

FIG. 2. ER stress induced by TPG increases the expression of fetuin-A. TPG induced fetuin-A expression in a dose-dependent (A and C) and time-dependent manner (B and D). HepG2 cells were treated with various doses of TPG for 12 h (A and C). Cells were then harvested for the detection of fetuin-A mRNA by quantitative real-time PCR (A and B) or protein expression by Western blot analysis (C and D). *, $P < 0.05$ and **, $P < 0.01$ as compared with the control group. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

SP600125, 4-phenylbutyrate (4-PBA) (Sigma-Aldrich, St. Louis, MO) for 30 min before treatment with the ER stress activator, thapsigargin (TPG) (Sigma-Aldrich), 25 mM glucose, and 1 mM palmitate (Sigma-Aldrich) at various intervals for Western blot analysis.

RNA extraction

Isolation of RNA from HepG2 cells was carried out with a commercial reagent (TRIzol; Life Technologies, Inc., Carlsbad, CA). After phenol/chloroform extraction and isopropanol precipitation, the RNA samples were washed with 75% ethanol and then quantified using a spectrophotometer (Gene Quant II; Amersham Pharmacia Biotech, Inc., Cambridge, UK) at 260 nm and stored at −80 C.

Quantitative real-time PCR

All subsequent quantification steps were performed according to the manufacturer's instructions. Briefly, single-stranded cDNA were synthesized from 5 μg of total RNA using 25 U of reverse transcriptase (Promega, Madison, WI) and 6 μg/ml of oligo primers (Promega) at 37 C for 2 h. PCR amplification was performed in a final 20-μl volume consisting of 5 μl of cDNA, 0.5 μM sense and antisense primers, 0.2 μM fluorescence probes, 4 mM $MgCl_2$, and 2 μl of FastStart Taq DNA polymerase (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Each sample (20 μl) was placed in a thermocycler glass capillary. Temperature cycling for the PCR run comprised denaturation at 95 C for 10 min, the annealing conditions at 95 C for 5 sec, 55 C for 10 sec, an extension at 72 C for 15 sec (50 cycles), and a cooling step to 40 C. Real-time PCR was monitored online using a thermocycler (LightCycler 2.0; Roche Molecular Systems, Alameda, CA). The oligonucleotide primers of fetuin-A and glyceraldehyde-3-phosphate dehydrogenase were the same as those used in a previous study (18). The reaction product of glyceraldehyde-3-phosphate dehydrogenase served as a reference to normalize each sample for relative quantification.

Western blot analyses

The samples were lysed with a buffer (pH 7.5) containing 450 mM NaCl, 3% Nonidet P-40, 1.5% sodium deoxycholate, 0.3% sodium dodecyl sulfate, 150 mM Tris-HCl (pH 8.0), and 3 mM EDTA, with proteinase and phosphotase inhibitor cocktail (Sigma-Aldrich). The supernatant was collected and the protein levels were quantified with a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). The cell lysates were then boiled in sample buffer for 5 min. Protein (30 μg) from the lysates was resolved by SDS-PAGE (10% gels) and transferred on to polyvinylidene fluoride membranes (Millipore, Billerica, MA). After blocking with 10 mM Tris (pH 7.6), 150 mM NaCl, and 0.05% Tween 20 containing 10% skim milk at room temperature for 1 h, the blots were probed with primary antibodies, such as phospho-ERK1/2, phospho-p38, phospho-c-Jun N-terminal kinase (JNK), fetuin-A, ERK1/2, p38, JNK, and actin (Signaling Technology, Beverly, MA) at 4 C overnight. After the membranes had been washed with 10 mM Tris (pH 7.6), 150 mM NaCl, and 0.05% Tween 20, the blots were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The protein bands were detected using Immobilon (Millipore). The optical density of the protein levels was determined using VisionWorks LS software (Upland, CA).

Animals

C57BL/6J male mice were purchased from the Animal Center of National Cheng Kung University Medical College, and the animal experiments were conducted in accordance with Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the guidelines of the Animal Welfare Act. Insulin-deficient diabetic mice were induced by ip injection of 65 mg/kg STZ (Sigma-Aldrich) after starvation for 4 h during five consecutive days. Although most of the related works induce insulin-deficient diabetic mice with a single high-dose injection of STZ, the current study followed other research by using multiple low-dose STZ injections (31–33), and this method had a high success rate and low mortality of the animals. Phlorizin (Sigma, St. Louis, MO) was dissolved in a solution containing 10% ethanol, 15% dimethylsulfoxide, and 75% saline and was injected sc at a dose of 0.4 g/kg twice daily for 2 wk. Control mice were injected with the same volume of vehicle solution. Insulin-resistant diabetic mice were induced by feeding with a HFD containing 34.9% fat (wt/wt) for 12 wk (TestDiet, Richmond, IN) starting from 8 wk of age. 4-PBA is a low mo-

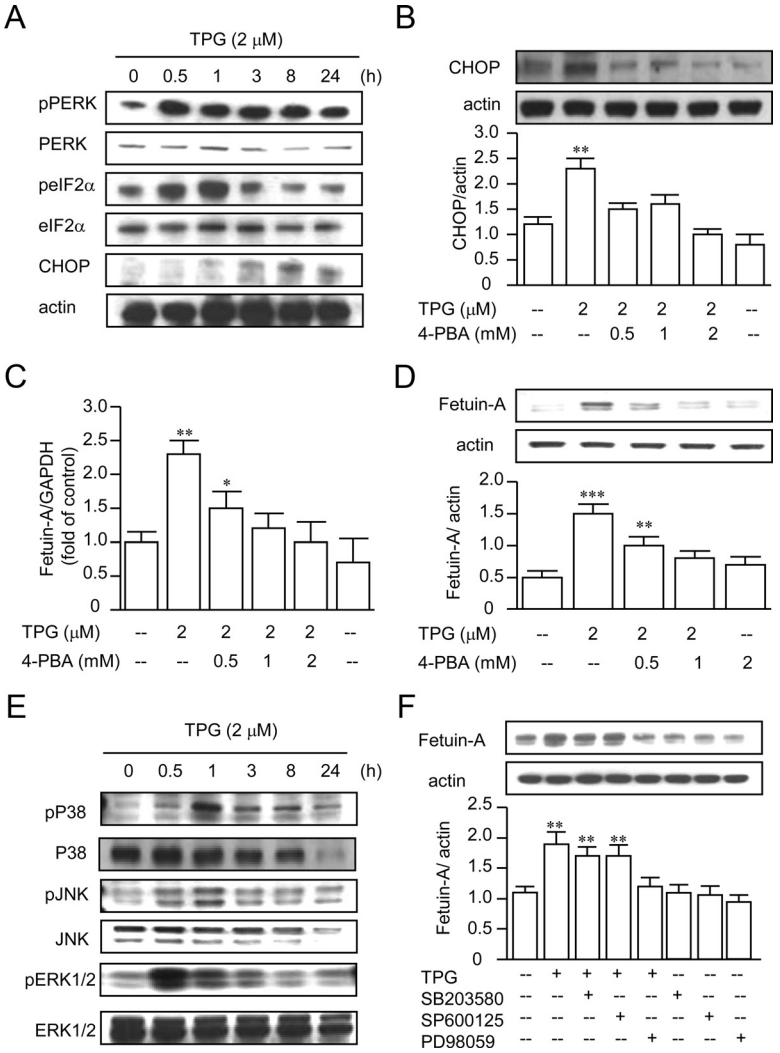

FIG. 3. ER stress induced the expression of fetuin-A through a ERK1/2-dependent pathway. A, Effects of TPG on ER stress-related protein activation. Cells were treated with 2 μM TPG and harvested at indicated periods. B, C, and D, Effects of ER stress inhibitor, 4-PBA, on TPG-induced fetuin-A mRNA (C) or protein expression (D). Cells were treated with various doses of 4-PBA for 1 h before TPG treatment for 12 h. Cell lysates were then harvested for the detection of CHOP (B) or fetuin-A (D) protein levels, and mRNA expression of fetuin-A (C). E, HepG2 cells were treated with 2 μM TPG, and the cells were harvested at the indicated periods for the detection of the phosphorylation of MAPK by Western blot analysis. F, HepG2 cells were pretreated with 25 μM MAPK inhibitors, and the cells were harvested at 12 h for the detection of fetuin-A expression by Western blot analysis. The OD of Western blottings was assessed using VisionWorks LS software. Data are expressed as mean ± SEM obtained from three individual cultures. *, $P < 0.05$ and **, $P < 0.01$ as compared with the control group. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

lecular weight compound known to stabilize protein conformation, improve ER folding capacity, and facilitate the trafficking of mutant proteins (34). Previous studies show that 4-PBA significantly inhibits ER stress-related signals in cell model (28, 35), and treatment with 4-PBA in ob/ob mice markedly reduces protein kinase R-like ER kinase (PERK) and inositol-requiring enzyme-1α phosphorylation in the liver (28). 4-PBA was dissolved in PBS by titration with 5 N NaOH. One gram per kilogram 4-PBA was given in mice by daily gavage for 3 wk following the procedure in a previous study (36). The data were obtained from at least eight mice from each group.

Intraperitoneal GTT (IPGTT) and insulin tolerance test (ITT)

Both IPGTT and ITT were performed in HFD-induced insulin-resistant diabetic mice after an 8-h fast, as previously described (37). IPGTT was performed by ip injection of 1 g/kg glucose. ITT was carried out by ip administration of 1.0 mU/g insulin (Novo Nordisk, Bagsvaerd, Denmark). The blood samples were collected at 0, 30, 60, 90, and 120 min from the retro-orbital sinus of each mouse after injection of glucose or insulin. The blood samples were then centrifuged at 12,000 × *g* for 5 min. The plasma blood glucose levels were determined using a commercial assay kit (Bio-Systems, Barcelona, Spain).

Blood biochemistry

Whole blood collected from the retro-orbital venous sinus was captured using sodium-heparinized microhaematocrit capillary tubes (Marienfeld-Superior, Lauda-Königshofen, Germany) and then transferred to Eppendorf tubes and then centrifuged at 12,000 × *g* for 5 min. Blood glucose concentrations were measured with a glucose kit (Biosystems SA, Barcelona, Spain) by an automatic blood glucose meter (Biosystem BTS-330; Biochemistry Analyzer, Barcelona, Spain). ELISA kits (Mercodia AB) were used to determine serum insulin levels. Other biochemical variables were assessed with an automatic blood chemistry analyzer Dry-Chem 4000i (Fujifilm, Saitama, Japan). Using reagents obtained from the instrument manufacturer, triglyceride, cholesterol, blood urea nitrogen concentrations, ALT, and AST levels were determined.

Statistical analysis

SPSS software (version 17.0; SPSS, Chicago, IL) was used for the statistical analysis. All normally distributed continuous variables were expressed as means ± SD or mean ± SEM. Study subjects were divided into four groups: NGT, NDD, NAFLD, and NDD + NAFLD. ANOVA was used to compare the continuous variables among the subjects. Student's *t* tests or pair *t* tests were used for comparison of variables between groups. A *P* value less than 0.05 was considered statistically significant.

Results

The serum levels of fetuin-A were significantly increased in subjects with both diabetes and NAFLD

Table 1 shows the clinical characteristics of the study subjects. There were significant differences in body weight, waist circumference, BMI, systolic/diastolic blood pressure, fasting and postload 2-h plasma glucose, A1C, CRP, triglyceride, HDL cholesterol, LDL/HDL-cholesterol ratio, HOMA-IR, and adiponectin among subjects with NGT, NDD, NAFLD, and NDD + NAFLD. In gen-

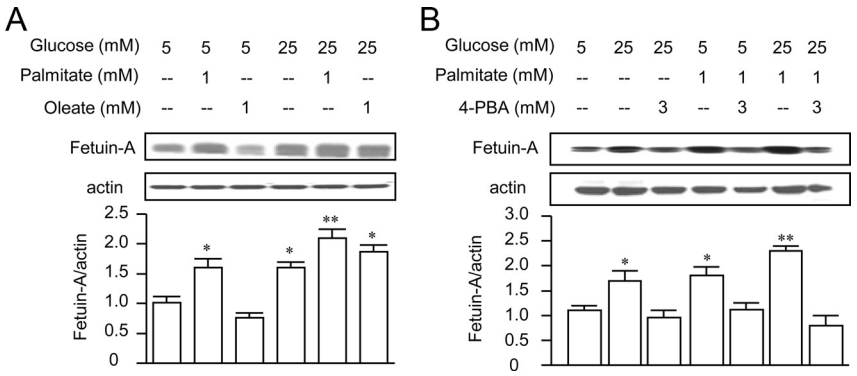

FIG. 4. Pharmacological inhibition of ER stress decreased high glucose and palmitate-induced fetuin-A expression. A, HepG2 cells were treated with 25 mM glucose, 1 mM palmitate or oleate, and harvested at 12 h. Cell lysates were harvested for the detection of fetuin-A levels by Western blot analysis. B, HepG2 cells were pretreated with 3 mM 4-PBA and then treated with 1 mM palmitate or 25 mM glucose. Cell lysates were harvested after 12 h of treatment for the detection of fetuin-A levels by Western blot analysis. Data are expressed as mean ± SEM obtained from three individual cultures. *, $P < 0.05$ and **, $P < 0.01$ as compared with the control group.

eral, subjects with NAFLD had higher body weight, BMI, and waist circumference than those without NAFLD, and NDD + NAFLD subjects had the highest BMI and waist circumference. Diabetic subjects had a higher fasting and postload plasma glucose and A1C levels than nondiabetic subjects, irrespective of the presence or absence of NAFLD. The CRP, triglyceride, and HOMA-IR levels in subjects with NDD or NAFLD were comparable and higher than those of NGT subjects but lower than those of the NDD + NAFLD subjects. However, the differences based on age, sex, ALT, AST, creatinine, eGFR, total cholesterol, and the LDL cholesterol were not significant.

The serum adiponectin levels decreased gradually from NGT, NDD, NAFLD, to NDD + NAFLD subject ($P < 0.001$ test for trend). In contrast, serum fetuin-A level increased gradually with the concentrations of 291 ± 63, 337 ± 96, 339 ± 84, and 385 ± 77 μg/ml in subjects with NGT, NDD, NAFLD, and NDD + NAFLD, respectively ($P < 0.001$ test for trend) (Fig. 1). NDD, NAFLD, and NDD + NAFLD subjects had significantly higher fetuin-A levels than the subjects with NGT ($P < 0.05$, 0.05, and 0.001, respectively). In addition, the fetuin-A concentration was also higher in subjects with NDD + NAFLD as compared with those of NDD ($P < 0.05$) and NAFLD ($P < 0.05$). However, NDD subjects had similar fetuin-A levels to those with NAFLD. The differences in fetuin-A concentrations between groups remained significant even after adjusting for age, gender, and BMI.

ER stress increased the expression of fetuin-A

TPG, the ER stress activator, was used to further investigate the role of ER stress in the expression of fetuin-A. Treatment with TPG in HepG2 cells increased both the mRNA (Fig. 2, A and B) and protein expression (Fig. 2, C and D) of fetuin-A in a dose- and time-dependent manner (Fig. 2). The core of ER stress is a triad of stress-sensing proteins, such as PERK, eukaryotic translation initiation factor 2α (eIF2α), and CCAAT enhancer binding protein homologous protein (CHOP) (38). Treatment with TPG in HepG2 cells significantly activated the ER stress-sensing proteins (Fig. 3A). To confirm the role of ER stress in the expression of fetuin-A, a ER stress inhibitor, 4-PBA,

TABLE 2. Clinical biochemistry data of the control and experimental diabetic mice

		HFD		STZ	
	Chow	4-PBA (−)	4-PBA (+)	4-PBA (−)	4-PBA (+)
Body weight (g)	28.4 ± 1.5	46.6 ± 4.2[c]	41.7 ± 3.6[c]	21.2 ± 3.2[b]	22.7 ± 2.6[b]
Plasma glucose (mg/dl)	132.8 ± 8.7	178.2 ± 11.2[b]	127.7 ± 10.6[e]	461.2 ± 21.5[c]	20.4 ± 0.6[c,d]
Insulin (μIU/ml)	16.7 ± 1.5	26.1 ± 1.3[b]	19.1 ± 2.7[e]		
ALT (U/liter)	43.8 ± 9.2	201.5 ± 22.9[b]	184.3 ± 18.5[b]	129.5 ± 17.8[c]	109.8 ± 11.4[c]
AST (U/liter)	71.4 ± 7.9	168.7 ± 11.8[b]	141.8 ± 16.7[b]	214.8 ± 35.5[c]	199.4 ± 15.8[c]
Creatinine (mg/dl)	0.5 ± 0.1	0.6 ± 0.1[b]	0.6 ± 0.1[b]	0.8 ± 0.1[b]	0.8 ± 0.1[b]
BUN (mg/dl)	44 ± 5	72 ± 2[b]	63 ± 6[b]	138 ± 13[c]	130 ± 19[c]
Cholesterol (mmol/liter)	4.5 ± 0.3	5.8 ± 0.3[a]	5.6 ± 0.4[a]	7.4 ± 0.4[c]	6.9 ± 0.6[c]
Triglyceride (mmol/liter)	0.8 ± 0.1	1.4 ± 0.1[b]	0.9 ± 0.1[e]	2.0 ± 0.2[c]	1.7 ± 0.1[c,d]

Chow, Normal chow-fed control mice; HFD, HFD-induced insulin-resistant diabetic mice; STZ, STZ-induced insulin-deficient diabetic mice; BUN, blood urea nitrogen.

[a] $P < 0.05$ compared with normal chow-fed control mice.

[b] $P < 0.01$ compared with normal chow-fed control mice.

[c] $P < 0.001$ compared with normal chow-fed control mice.

[d] $P < 0.01$ compared with 4-PBA (−) mice.

[e] $P < 0.001$ compared with 4-PBA (−) mice.

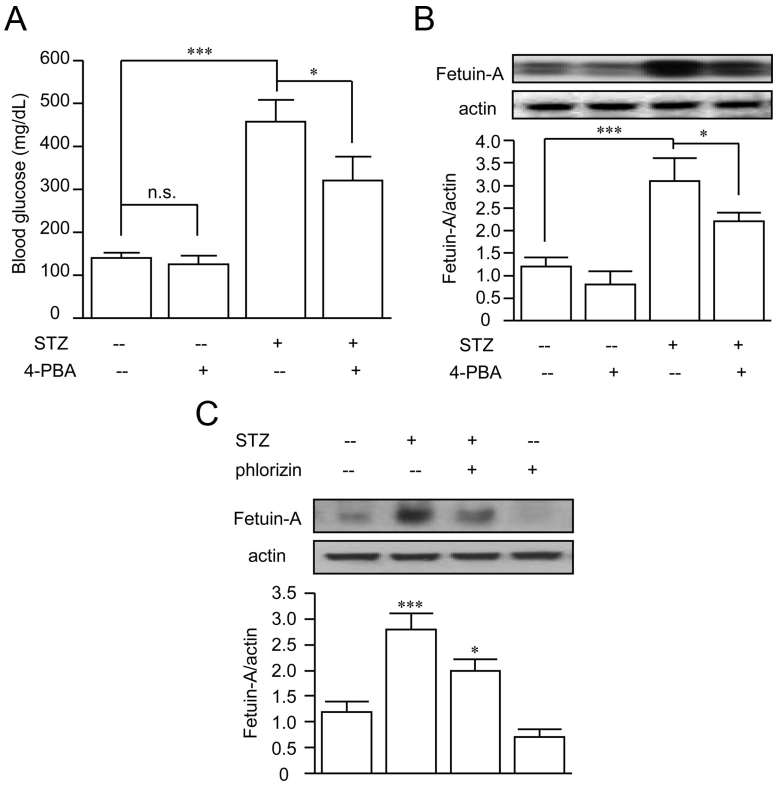

FIG. 5. Treatment with ER stress inhibitor significantly decreased the elevated hepatic fetuin-A expression in STZ-induced insulin-deficient diabetic mice. The STZ-induced insulin-deficient diabetic mice were treated with 1 g/kg 4-PBA for 3 wk and then fasted for 8 h for the determination of blood glucose levels (A), and the protein levels of fetuin-A were determined by Western blot analysis (B). The STZ-induced insulin-deficient diabetic mice were treated with phlorizin at a dose of 0.4 g/kg twice daily for 2 wk. The hepatic fetuin-A expression was then detected by Western blot analysis (C). *, $P < 0.05$ and ***, $P < 0.001$ as compared with the indicated group or control group. NS, Not significant.

was used. Pretreatment with 4-PBA in HepG2 cells significantly inhibited TPG-induced CHOP expression (Fig. 3B). In addition, TPG-induced mRNA (Fig. 3C) and protein expression (Fig. 3D) of fetuin-A were inhibited by 4-PBA, implying the involvement of ER stress in the regulation of fetuin-A expression. Moreover, TPG increased the phosphorylation of MAPK, including ERK1/2, JNK, and p38 (Fig. 3E). However, pretreatment with the ERK1/2 inhibitor, PD98059, significantly reversed TPG-induced fetuin-A expression, whereas pretreatment with the p38 and JNK inhibitors showed no significant effects. Pretreatment with MAPK inhibitors alone showed no effect on fetuin-A expression in HepG2 cells (Fig. 3F).

Inhibition of ER stress decreased high glucose and palmitate-induced fetuin-A expression

We further investigated the role of ER stress in high glucose- and palmitate-induced fetuin-A expression. The expression of fetuin-A was significantly increased in both HepG2 cells maintained in high glucose condition or treated with palmitate, whereas treatment with oleate showed no significant effect on fetuin-A expression (Fig. 4A). Moreover, the level of fetuin-A was significantly increased in palmitate-treated cells with high glucose, as compared with the palmitate-treated or high glucose-treated alone groups (Fig. 4A), implying that hepatic steatosis and diabetes may synergically increase the levels of fetuin-A. Moreover, inhibition of ER stress by chemical chaperones reversed both the high glucose- and palmitate-induced fetuin-A expression (Fig. 4B).

Administration of ER stress inhibitor in STZ-induced insulin-deficient diabetic mice decreased the expression of fetuin-A in liver

STZ-induced insulin-deficient diabetic mice were used to evaluate the role of ER stress in the regulation of fetuin-A expression *in vivo*. After administration of STZ in mice, not only were impaired liver and kidney function observed, but blood glucose, triglyceride, and cholesterol levels were also significantly increased as compared with the control group (Table 2). Treatment with ER stress inhibitor decreased triglyceride, whereas hepatic and renal function were not affected (Table 2). In addition, 4-PBA slightly but significantly decreased the blood glucose levels in STZ mice, whereas the effect was not observed in the control group (Fig. 5A). The level of fetuin-A in the liver was significantly increased in STZ-induced insulin-deficient diabetic mice, and treatment with 4-PBA reversed this effect (Fig. 5B). To investigate whether fetuin-A increased because of the higher glucose levels, or due to ER stress activation, the STZ-induced insulin-deficient diabetic mice were treated with phlorizin. The phlorizin partially reversed the increase of hepatic fetuin-A expression in STZ mice and slightly decreased expression of fetuin-A in wild-type mice (Fig. 5C).

Administration of ER stress inhibitor in HFD-induced insulin-resistant diabetic mice decreased the hepatic expression of fetuin-A and improved insulin sensitivity

The role of ER stress in the regulation of fetuin-A expression was further confirmed in HFD-induced insulin-resistant diabetic mice. Impaired hepatic and renal functions were observed in HFD-induced insulin-resistant diabetic mice, and blood glucose, triglyceride, and cholesterol levels were also significantly increased as compared with the control group (Table 2). Treatment with

ER stress inhibitor decreased triglyceride, whereas hepatic and renal functions were not affected (Table 2). In addition, 4-PBA significantly decreased the blood glucose levels in HFD-induced insulin-resistant diabetic mice, whereas the effect was not observed in the control group (Fig. 6). The hepatic levels of fetuin-A were significantly increased in HFD-induced insulin-resistant diabetic mice, and treatment with ER stress inhibitor significantly reversed the increased levels of fetuin-A, whereas this effect was not observed in the control group (Fig. 6A). Moreover, treatment with the ER stress inhibitor not only improved HFD-induced impaired glucose utility (Fig. 6B) but also ameliorated insulin resistance (Fig. 6C).

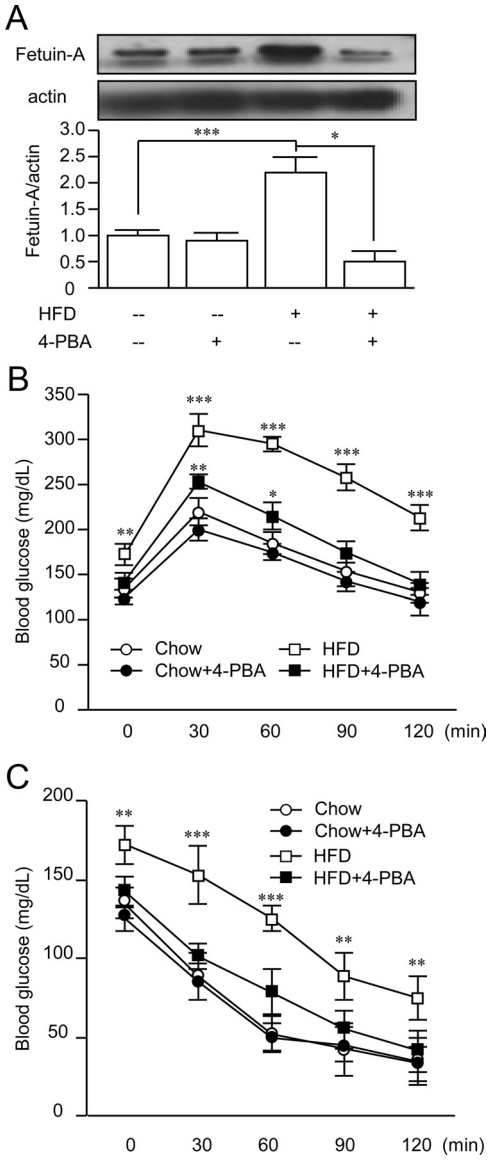

FIG. 6. Treatment with ER stress inhibitor significantly decreased the hepatic fetuin-A levels and improved glucose utility and insulin sensitivity in HFD-induced insulin-resistant diabetic mice. The HFD-induced insulin-resistant diabetic mice were treated with 1 g/kg 4-PBA for 3 wk. A, The mice in each group were fasted for 8 h, and then the liver samples were collected for the detection of fetuin-A levels. B, A GTT (1 g of glucose/kg) was performed in mice that fasted for 8 h. C, ITT was performed in fasted mice that received normal chow (*opened circles*), normal chow with 4-PBA treatment (*closed circles*), HFD-induced diabetic mice (*opened squares*), and 4-PBA-treated HFD-induced diabetic mice (*closed squares*) (n = 6–8). Data are expressed as mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with normal chow-fed mice.

Discussion

A close relationship between fetuin-A and excess substrate (such as glucose or fatty acid) presented to the liver has been found in human, animal, and cell studies. In humans, serum fetuin-A levels are positively associated with liver fat (7, 8) and diabetes (9); its expression is significantly elevated in HFD-fed mice with fatty liver (8) and diabetic mice with hyperlipidemia (18); and its secretion from human hepatoma cell line (HepG2) is enhanced by high glucose (22) and palmitate (18). The results of the present study show that the level of fetuin-A was significantly increased in both diabetic and NAFLD subjects, supporting the findings of previous works on fetuin-A in diabetic patients and animals. Moreover, the results of the current work also show that diabetic patients with NAFLD have significantly increased levels of fetuin-A compared with diabetic or NAFLD subjects (Fig. 1), implying that hyperglycemia and hepatic steatosis might synergically enhance the expression of fetuin-A. Both hyperglycemia and hyperlipidemia induce ER stress, and this contributes to the development of insulin resistance and diabetes (23–29). Therefore, this study further investigated the role of ER stress in the regulation of fetuin-A expression. Treatment with ER stress activator significantly increased the expression of fetuin-A in a dose- and time-dependent manner through an ERK1/2-dependent pathway (Figs. 2 and 3). Pretreatment with ER stress inhibitor blocked high glucose and palmitate-induced fetuin-A expression (Fig. 4). Furthermore, treatment with ER stress inhibitor in insulin-deficient (Fig. 5) or insulin-resistant diabetic mice (Fig. 5) not only decreased the serum levels of fetuin-A but also increased glucose utility and insulin sensitivity to regulate blood glucose.

Although an earlier study indicated that human or bovine fetuin-A show no robust inhibition of insulin signaling (39), another work showed that rat fetuin-A inhibits insulin receptor and downstream substrate phosphorylation signaling (16). Although fetuin-A has shown no classical insulin receptor inhibition in rat adipocytes, the unexplained down-regulation of insulin-stimulated Ets-like protein 1-phosphorylation has been observed (39). Although the effects of fetuin-A on the phosphorylation of insulin receptors remain controver-

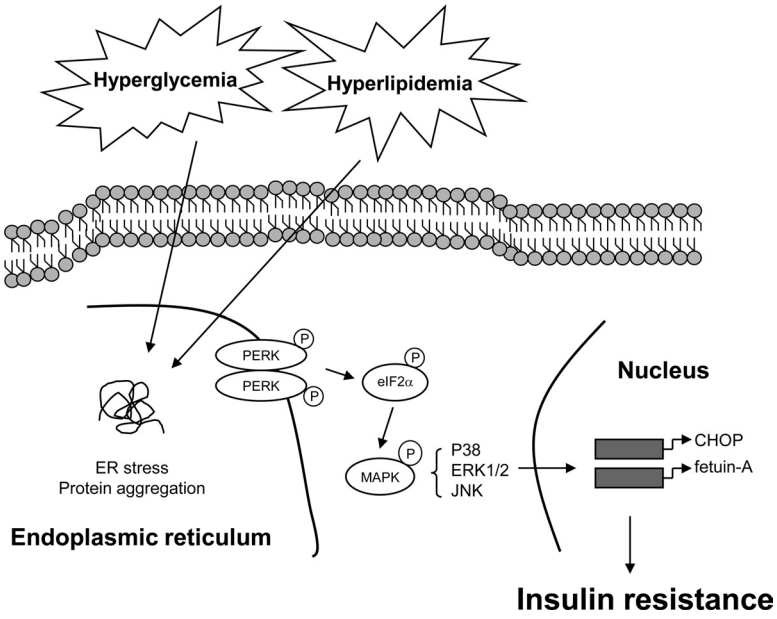

FIG. 7. Hypothetical scheme of the role of ER stress in the regulation of fetuin-A expression. In this study, we provide evidence to clarify the regulation of fetuin-A expression. Both hyperlipidemia and hyperglycemia induce protein misfolding to increase ER stress and further activate the phosphorylation of PERK, eIF2α, and MAPKs. Activation of ERK1/2 increases the expression of fetuin-A levels in hepatic cells and the increase of fetuin-A correlated with the development of insulin resistance in type 2 diabetic animals or patients.

sial, it is possible that it inhibited other insulin substrates (39). On the basis of the previous reports, it is reasonable to assume that fetuin-A is an important factor associated with insulin resistance and type 2 diabetes (15–22). Although it is well known that the levels of fetuin-A are highly correlated with diabetes (9) and hepatosteatosis (8), the current study is the first report demonstrating that diabetic patients with NAFLD have a higher level of plasma fetuin-A as compared with diabetic or NAFLD patients (Fig. 1).

Previous studies indicated that high levels of glucose (22) and palmitate (18) induce the expression of fetuin-A through activation of the ERK1/2 signaling pathway (22) and binding of nuclear factor κB (18), respectively. However, the common pathways between glucose toxicity and lipotoxicity that regulate fetuin-A expression are still unknown. Both glucose toxicity and lipotoxicity increase ER stress, and activation of ER stress contributes to the development of insulin resistance and diabetes (29). In the present study, treatment with ER stress activator in HepG2 cells significantly up-regulated the expression of fetuin-A (Fig. 2), and the ER stress-related proteins were also activated (Fig. 3A). Pretreatment with ER stress inhibitor blocked the increase of fetuin-A, indicating the involvement of ER stress in the regulation of fetuin-A (Fig. 3, C and D). A previous study indicated that phosphorylation of ERK1/2 enhances fetuin-A promoter activity to increase fetuin-A expression (22). Therefore, the current work further investigated the role of MAPK in the regulation of fetuin-A expression. Consistent with the findings of a previous study (40), TPG stimulated dual phosphorylation of JNK, p38, and ERK MAPK (Fig. 3E). However, only pretreatment with ERK1/2 inhibitor, PD98059, blocked TPG-induced fetuin-A expression (Fig. 3F), indicating the involvement of ERK1/2 in the regulation of fetuin-A expression. Furthermore, increased fetuin-A expressions induced by high levels of glucose and palmitate were both blocked by the ER stress inhibitor, implying that ER stress is the common pathway between glucotoxicity and lipotoxicity that regulates fetuin-A expression (Fig. 4).

Although it has been reported that 4-PBA has no beneficial effects on blood glucose in alloxan-induced insulin-deficient diabetic mice, a longer treatment of 3 wk with 4-PBA showed a significant glucose-lowering effect in STZ-induced diabetic mice (Fig. 5A). In addition, the hepatic fetuin-A levels were decreased after treatment with the ER stress inhibitor (Fig. 5B). Moreover, an earlier work demonstrated a reduction in ER stress caused by 4-PBA restored glucose homeostasis in insulin-resistant diabetic mice (28), whereas increased levels of fetuin-A were observed in insulin-resistant diabetic rats (18). After treatment with 4-PBA in the current work, hepatic fetuin-A expression decreased (Fig. 6A) and the glucose utility (Fig. 6B) and insulin sensitivity (Fig. 6C) increased in HFD-induced insulin-resistant diabetic animals.

Taken together, the results of this study show that the level of fetuin-A was increased in diabetic patients who also had NAFLD. Moreover, ER stress induced by high levels of glucose and palmitate also increased the expression of fetuin-A and further contributed to the development of insulin resistance. Both hyperlipidemia and hyperglycemia induce protein misfolding, which increases ER stress and further activates the phosphorylation of PERK, eIF2α, and MAPK. Activation of ERK1/2 increases the expression of fetuin-A levels in hepatic cells, and the increase in fetuin-A is correlated with the development of insulin resistance in type 2 diabetic animals or patients (Fig. 7). The *in vitro* studies of cell lines in this work provide associative data that can only be used to clarify the possible mechanism of fetuin-A regulation, and the results suggest that hyperglycemia or NAFLD may not be the only factors that regulate it. Therefore, further studies are re-

quired to investigate the detailed regulation of fetuin-A expression.

Acknowledgments

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