Involvement of Endoplasmic Reticulum Stress in Insulin Resistance and Diabetes*

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Type 2 diabetes is one of the most prevalent and serious metabolic diseases in the world, and insulin resistance and pancreatic β-cell dysfunction are the hallmarks of the disease. In this study, we have shown that endoplasmic reticulum (ER) stress, which is provoked under diabetic conditions, plays a crucial role in the insulin resistance found in diabetes by modifying the expression of oxygen-regulated protein 150 (ORP150), a molecular chaperone that protects cells from ER stress. Sense ORP overexpression in the liver of obese diabetic mice significantly improved insulin resistance and markedly ameliorated glucose tolerance. Conversely, expression of antisense ORP150 in the liver of normal mice decreased insulin sensitivity. The phosphorylation state of IRS-1 and Akt, which are key molecules for insulin signaling, and the expression levels of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, key enzymes of gluconeogenesis, were also altered by ORP150 overexpression. This is the first report showing that ER stress plays a crucial role in the insulin resistance found in diabetes and thus could be a potential therapeutic target for diabetes.

Type 2 diabetes is one of the most prevalent and serious metabolic diseases in the world, and insulin resistance and pancreatic β-cell dysfunction are the hallmarks of the disease (1, 2). Normal 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, insulin resistance is further increased and β-cell function progressively deteriorates. The significance of hyperglycemia as a direct cause of these phenomena has been called “glucose toxicity” (3, 4).

The endoplasmic reticulum (ER) is an organelle that synthesizes various secretory and membrane proteins. These proteins are correctly folded and assembled by chaperones in the ER. During stressful conditions, such as an increase in the misfolded protein level, the chaperones become overloaded and the ER fails to fold and export newly synthesized proteins, leading to ER stress (5–9). We hypothesized that the ER stress provoked in diabetes is involved in various phenoma found in the disease. It has indeed previously been shown that ER stress is involved in pancreatic β-cell dysfunction (10–14). Oxygen-regulated protein 150 (ORP150), a molecular chaperone found in the ER, has been shown to protect cells from ER stress (15, 16). Here we report that ORP150 overexpression markedly improves insulin resistance and ameliorates glucose tolerance in diabetic animals, indicating that ER stress plays a crucial role in insulin resistance and could be a potential therapeutic target for diabetes.

MATERIALS AND METHODS

Preparation of Recombinant Adenoviruses—Recombinant adenoviruses expressing sense ORP (Ad-S-ORP) and antisense ORP (Ad-AS-ORP) were prepared, and the adenovirus titers were increased up to 1 × 10⁹ pfu/ml in the 293 cells. Adenovirus titers were further increased up to 1 × 10¹⁰ pfu/ml using Adeno-X virus purification kit (Clontech). Control adenovirus expressing green fluorescent protein (Ad-GFP) was also prepared in the same manner. Virus titers were estimated using an Adeno-X virus titer kit (Clontech).

Animals and Administration of Recombinant Adenoviruses—Male C57BL6 and C57BL/KsJ-db/db mice were purchased from Japan SLC. Mice (8 weeks old) were injected with Ad-S-ORP, Ad-AS-ORP, or Ad-GFP (1 × 10¹⁰ pfu/ml for Ad-S-ORP and 2 × 10¹⁰ pfu/ml for Ad-AS-ORP) from the cervical vein. After adenovirus injection, blood glucose levels were measured regularly with a portable glucose meter (Glu-test Sensor; Sanwa) after tail snipping. For measurement of serum insulin levels, blood samples of mice after a 6-h fast were collected into heparinized capillary tubes and serum insulin levels were determined with an insulin-EIA test kit (Glazyme).

Glucose Tolerance Tests—After a 6-h fast, mice were injected intraperitoneally with glucose (2.0 g/kg body weight). Blood samples were taken at various time points (0–120 min), and blood glucose levels and serum insulin levels were determined as described above.

Insulin Tolerance Tests—After a 6-h fast, mice were injected intraperitoneally with insulin (2.0 units/kg for C57BL/KsJ-db/db mice). Blood samples were taken at various time points (0–90 min), and blood glucose levels were measured as described above.

Euglycemic Hyperinsulinemic Clamp—Fourteen days before the clamp study, Ad-S-ORP (1 × 10¹⁰ pfu/ml) or Ad-AS-ORP (2 × 10¹⁰ pfu/ml) was injected from the left jugular vein. Three days before the clamp study, a silicon catheter (Phicon tube; Fuji-Systems) was inserted into the right jugular vein under general anesthesia with sodium pentobarbital. The catheter, which is required for infusion in the clamp study, was exteriorized at the back of the neck through a subcutaneous tunnel and filled with heparinized saline (200 units/ml). Clamp studies were performed on mice under conscious and unstressed conditions after a 6-h fast. A euglycemic hyperinsulinemic clamp with a tracer dilution method was applied to determine peripheral glucose uptake and endogenous glucose production. Experiments consisted of a 120-min euglycemic hyperinsulinemic clamp period (15 pmol/kg/min of regular human insulin for C57BL6 mice, 27 pmol/kg/min for C57BL/KsJ-
db/db mice during the 120-min clamp period). During this period, blood glucose levels were monitored every 5 min and the rate of 50% glucose containing 10% [6,6-2H2]glucose infusion into the jugular vein was adjusted to maintain blood glucose concentrations at 120 ± 10 mg/dl.

**Measurement of Endogenous Hepatic Glucose (HGP) Production by Stable Isotope-Labeled Glucose Enrichment**—To estimate HGP, stable isotope-labeled glucose enrichment was determined. Blood samples were taken at 90, 105, and 120 min, and 20 μl of each plasma sample were deproteinized with 60 μl of 95.9% ethanol. The supernatant was evaporated, and the residue was derivatized by the following procedure. First, 7.5 μl of MBTFA (N-methyl-bis(trifluoroacetamide); Pierce) and 7.5 μl of pyridine were added to the residue, and the mixture was heated for 1 h at 60 °C. The reaction product (1 μl) containing trifluoroacetylated glucose was then analyzed by gas chromatography and mass spectrometry (Model TSQ-700; Finningan-MAT) with a silicon S.E.-30 capillary column (30 m × 0.25 mm; Gasukuro Kogyo). The trifluoroacetyl derivative of glucose was separated from the other compounds by gas chromatography and was analyzed by electron impact mass spectrometry at 70 eV. The fragment ion peaks of unlabeled and [6,6-2H2]glucose were measured at a mass/electrical charge of 319 and 321, respectively.

**Western Blot Analysis and Immunoprecipitation**—Whole cell extracts obtained from liver were fractionated by 10% SDS-PAGE and transferred to reinforced cellulose nitrate membrane (Optitran BA-S85; Schleicher & Schuell). After blocking, the membranes were incubated at 4 °C overnight in TBS buffer (50 mM Tris-HCl, 150 mM NaCl) containing 1:1000 dilution of rabbit anti-IRS-1 antibody (Upstate Biotechnology), anti-Akt, and anti-Akt-pSer473 antibody (Cell Signaling) and then incubated for 1 h at room temperature in TBS containing 1:1000 dilution of anti-rabbit IgG antibody coupled to horseradish peroxidase (Bio-Rad). In addition, expression of the tyrosine-phosphorylated form of IRS-1 was examined by immunoprecipitation using anti-IRS-1 and anti-phosphotyrosine antibody (Upstate Biotechnology). Immunoreactive bands were visualized by incubation with LumiGLO (Cell Signaling) and exposed to light-sensitive film.

**RESULTS AND DISCUSSION**

**ORP150 Overexpression in the Liver Markedly Reduces Insulin Resistance and Ameliorates Glucose Tolerance in Obese Diabetic C57BL/KsJ-db/db Mice**—First, to examine whether ER stress is increased in the liver under diabetic conditions, we evaluated ER stress levels in the livers of 10-week-old obese diabetic C57BL/KsJ-db/db mice. Expression levels of KDEL and Bip, both of which are ER stress markers, were much higher in the obese diabetic mice compared with 10-week-old non-diabetic C57BL6 mice (Fig. 1A), indicating that ER stress is actually increased under diabetic conditions. To examine the effect of sense ORP150 overexpression on insulin resistance and diabetes, we prepared sense ORP150 expressing Ad-S-ORP (1 × 1010 pfu/ml) and a GFP-expressing control adenovirus (Ad-GFP) and delivered each adenovirus to C57BL/KsJ-db/db obese diabetic mice from the cervical vein. By Western blot analysis, we confirmed an increase in ORP150 expression in the liver upon adenovirus injection (Fig. 1B), but not in other tissues such as muscle and adipose tissue (data not shown). In addition, expression levels of KDEL (GRP78/94) and Bip (GRP78) in Ad-S-ORP-treated mice were lower compared with those in Ad-GFP-treated db/db mice, indicating that ORP150 is actually acting to decrease ER stress in the liver (Fig. 1B). There was no difference in body weight and food intake between Ad-S-ORP-treated and Ad-GFP-treated db/db mice (data not shown). When C57BL/KsJ-db/db mice were treated with Ad-S-ORP, nonfasting blood glucose levels were markedly reduced (Fig. 2A), whereas no such effects were observed in Ad-GFP-treated mice (Fig. 2A) or in C57BL6 mice treated with Ad-S-ORP (data not shown). Fasting blood glucose concentrations (after a 6-h fast) were also significantly lower in Ad-S-ORP-treated mice compared with Ad-GFP-treated mice, although there was no difference in plasma insulin concentrations between the two groups (Fig. 2B). To examine the effects of ORP150 overexpression in the liver on insulin resistance, we performed the intraperitoneal insulin tolerance test. The hypoglycemic response to insulin was larger in Ad-S-ORP-treated C57BL/KsJ-db/db mice than in Ad-GFP-treated mice (Fig. 2C).

To investigate this point further, we performed the euglycemic hyperinsulinemic clamp test. Clamp studies were performed on mice under conscious and unstressed conditions after a 6-h fast. Experiments consisted of a 120-min euglycemic hyperinsulinemic clamp period (15 pmol/kg/min of regular human insulin for C57BL6 mice, 27 pmol/kg/min for C57BL/KsJ-db/db mice). Blood glucose levels were monitored every 5 min, and the rate of infusion of a 50% glucose solution containing 20% [6, 6-2H2]glucose into the jugular vein was adjusted to maintain...
blood glucose concentrations at 110 ± 10 mg/dl. The glucose infusion rates (GIR) of Ad-S-ORP-treated mice were significantly higher compared with Ad-GFP-treated mice (17.9 ± 3.7 versus 10.0 ± 2.8 mg/kg/min, \( p < 0.05 \)) (Fig. 2D), indicating that ORP150 overexpression in the liver reduces insulin resistance and thus ameliorates glucose tolerance in C57BL/KsJ-db/db mice. We also evaluated endogenous hepatic glucose production (HGP) in Ad-S-ORP-treated mice using tracer methods. HGP was significantly lower in Ad-S-ORP-treated mice compared with Ad-GFP-treated mice (9.5 ± 3.0 versus 18.0 ± 4.5 mg/kg/min, \( p < 0.05 \)) (Fig. 2D). These results indicate that the reduction of insulin resistance and amelioration of glucose tolerance by Ad-S-ORP overexpression are mainly because of the suppression of HGP.

**Antisense ORP150 Overexpression in the Liver Decreases Insulin Sensitivity in Non-diabetic C57BL6 Mice**—Next, to examine the effects of antisense ORP150 expression in the liver on insulin sensitivity and glucose tolerance in non-diabetic animals, we prepared an antisense ORP150-expressing adenovirus \( (2 \times 10^9 \text{ pfu/ml}) \) and a control adenovirus (Ad-GFP) and delivered each adenovirus to 8-week-old C57BL6 mice from the cervical vein. By Western blot analysis we confirmed a decrease in ORP150 expression in the liver upon adenovirus injection (Fig. 1B). In addition, expression levels of KDEL in Ad-AS-ORP-treated mice were higher than those in Ad-GFP-treated C57BL6 mice, although there was no difference in Bip expression levels (Fig. 1B). There was no difference in body weight and food intake between the two groups (data not shown). In addition, although there was no difference in nonfasting blood glucose levels (Fig. 3A) and in fasting blood glucose and serum insulin levels between Ad-AS-ORP-treated and Ad-GFP-treated mice (Fig. 3B), the intraperitoneal glucose tolerance test revealed that glucose tolerance is markedly worsened upon antisense ORP150 expression (Fig. 3C). Furthermore, in the euglycemic hyperinsulinemic clamp study, the GIR of Ad-AS-ORP-treated C57BL6 mice were significantly lower compared with Ad-GFP-treated mice (34.3 ± 3.5 versus 64.4 ± 6.3 mg/kg/min, \( p < 0.05 \)) (Fig. 3D), indicating that ER stress in the liver reduces insulin sensitivity in C57BL6 mice. Furthermore, we evaluated HGP in Ad-AS-ORP-treated mice using tracer methods. HGP in Ad-AS-ORP-treated mice was significantly greater than in Ad-GFP-treated mice (18.6 ± 2.1 versus 5.8 ± 2.0 mg/kg/min, \( p < 0.05 \)) (Fig. 3D). These results indicate that antisense ORP150 expression decreases insulin sensitivity at least in part by increasing HGP in non-diabetic mice.

**ER Stress Involved in Insulin Resistance and Diabetes**
FIG. 3. Effects of adenoviral antisense ORP overexpression in the liver on insulin resistance and glucose tolerance in C57BL6 mice. 

A, nonfasting blood glucose levels in C57BL6 mice treated with Ad-AS-ORP or Ad-GFP (n = 6). B, fasting blood glucose and serum insulin levels in C57BL6 mice 2 weeks after injection with Ad-AS-ORP or Ad-GFP (n = 6). C, glucose tolerance in C57BL6 mice treated with Ad-AS-ORP or Ad-GFP. Two weeks after the adenovirus injection, intraperitoneal glucose tolerance tests (IPGTT) were performed. After a 6-h fast, glucose was injected intraperitoneally at a dose of 2.0 g/kg body weight, and blood glucose levels were measured (*, p < 0.05, n = 6). D, glucose infusion rate and endogenous hepatic glucose production in C57BL6 mice treated with Ad-AS-ORP or Ad-GFP. Two weeks after the adenovirus injection, glucose infusion rate and hepatic glucose production were estimated by a euglycemic hyperinsulinemic clamp test (*, p < 0.05, n = 6).

FIG. 4. Effects of sense and antisense ORP overexpression on insulin signaling and gluconeogenesis in the liver. 

A, effects of ER stress on insulin signaling. C57BL/KsJ-db/db mice were treated with Ad-S-ORP or Ad-GFP, and C57BL6 mice were treated with Ad-AS-ORP or Ad-GFP. Two weeks after the adenovirus injection, the expression of total and tyrosine-phosphorylated forms of IRS-1 and total and phosphorylated forms (Ser-473) of Akt were examined by Western blot analysis. Similar results were obtained in three independent experiments. B, effects of ER stress on gluconeogenesis. C57BL/KsJ-db/db mice were treated with Ad-S-ORP or Ad-GFP, and C57BL6 mice were treated with Ad-AS-ORP or Ad-GFP. Two weeks after the adenovirus injection, mRNA levels of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, key enzymes of gluconeogenesis, were examined by Northern blot analysis. Similar results were obtained in three independent experiments.
for insulin signaling. IRS-1 tyrosine phosphorylation was markedly increased in Ad-S-ORP-treated C57BL/KsJ-db/db mice compared with Ad-GFP-treated mice (Fig. 4A). Concomitantly, an increase in Akt serine 473 phosphorylation was observed in Ad-S-ORP-treated C57BL/KsJ-db/db mice compared with Ad-GFP-treated mice (Fig. 4A). In contrast, IRS-1 tyrosine phosphorylation was decreased in Ad-AS-ORP-treated mice compared with Ad-GFP-treated mice (Fig. 4A). A decrease in Akt serine 473 phosphorylation was observed in Ad-AS-ORP-treated C57BL6 mice compared with Ad-GFP-treated mice (Fig. 4A). We next examined the expression levels of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), both of which are known to be regulated by insulin signaling. The expression of both PEPCK and G6Pase was markedly decreased by Ad-S-ORP treatment in C57BL/KsJ-db/db mice (Fig. 4B). In contrast, expression of both enzymes was increased upon treatment with Ad-AS-ORP in C57BL6 mice (Fig. 4B). These results indicate that reduction of ER stress enhances insulin signaling, which leads to a decrease in gluconeogenesis and amelioration of glucose tolerance.

In conclusion, sense ORP150 overexpression decreased insulin resistance and markedly improved glycemic control in diabetic model animals; in contrast, antisense ORP150 expression induced insulin resistance in non-diabetic control mice, indicating that ER stress plays a crucial role in the insulin resistance found in diabetes. ER stress could thus be a potential therapeutic target for diabetes.

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Page 850, Fig. 4: Although the legend is correct, the panels shown in Fig. 4 were incorrect. The correct panels are shown below.

![Corrected Figure 4](image_url)

Fig. 4

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