

# The Endoplasmic Reticulum Chaperone Improves Insulin Resistance in Type 2 Diabetes

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**To determine the role of the endoplasmic reticulum (ER) in diabetes, Akita mice, a mouse model of type 2 diabetes, were mated with either heterozygous knockout mice or two types of transgenic mice of 150-kDa oxygen-regulated protein (ORP150), a molecular chaperone located in the ER. Systemic expression of ORP150 in Akita mice improves insulin intolerance, whereas the exclusive overexpression of ORP150 in pancreatic  $\beta$ -cells of Akita mice did not change their glucose tolerance. Both an insulin tolerance test and hyperinsulinemic-euglycemic clamp revealed that ORP150 enhanced glucose uptake, accompanied by suppression of oxidized protein. Furthermore, ORP150 enhanced the insulin sensitivity of myoblast cells treated with hydrogen peroxide. These data suggest that ORP150 plays an important role in insulin sensitivity and is a potential target for the treatment of diabetes. *Diabetes* 54:657–663, 2005**

**H**yperglycemia occurs with the progressive failure of pancreatic  $\beta$ -cells to secrete sufficient amounts of insulin to compensate for insulin resistance (1). Mice lacking PKR-like endoplasmic reticulum kinase (PERK) or eukaryotic initiation factor (eIF)-2 $\alpha$  exhibited  $\beta$ -cell overload in pancreatic  $\beta$ -cells (2,3), which is observed during conditions such as

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ALA,  $\alpha$ -lipoic acid; CAG, chicken  $\beta$ -actin; Chop, C/EBP homologous protein; eIF, eukaryotic initiation factor; GRP, glucose-regulated protein; ER, endoplasmic reticulum; IPGTT, intraperitoneal glucose tolerance test; IRS, insulin receptor substrate; ITT, insulin tolerance test; ORP150, 150-kDa oxygen-regulated protein; PERK, PKR-like ER kinase; TM, tunicamycin.

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hyperglycemia and obesity. Furthermore, nitric oxide induces apoptosis by endoplasmic reticulum (ER) stress via the induction of C/EBP homologous protein (Chop), and pancreatic islets from Chop knockout mice exhibit resistance to nitric oxide (4). The Akita mouse, which carries a conformation-altering missense mutation (Cys96Tyr) in Insulin 2, displays hyperglycemia without obesity (5,6). During the development of diabetes in Akita mice, both the transcriptional factor Chop and the molecular chaperone GRP78 in the ER were induced in the pancreas, and targeted disruption of the Chop gene improved the glucose intolerance of heterozygous Akita mice (7). These reports show that ER plays an important role in insulin secretion in  $\beta$ -cells of the pancreas.

ORP150 (150-kDa oxygen-regulated protein) is a molecular chaperone in the ER that has been identified in cultured astrocytes exposed to hypoxia (8). The expression of ORP150 is essential for the maintenance of cellular viability under hypoxia (9), and neurons overexpressing ORP150 resist acute ischemic damage (10). ORP150 also plays an important role in the secretion of vascular endothelial growth factor (VEGF) as a molecular chaperone (11,12), and it is induced via the unfolded protein pathway (13). Kobayashi et al. (14) showed that the strong expression of ORP150 protein by islets of pancreas tissue is reduced by fasting, suggesting that ORP150 is involved in the secretion of insulin. In contrast, polymorphism analysis revealed that some single nucleotide polymorphisms (SNPs) in the ORP150 genome of Pima Indians are associated with insulin sensitivity and not the secretion of insulin (15).

In this report, we show that the systemic overexpression of ORP150 delayed the onset of disease in heterozygous Akita mice and improved insulin sensitivity, whereas heterozygous disruption of the ORP150 gene facilitated the progress of diabetes and caused insulin resistance. Furthermore, the overexpression of ORP150 reduced oxidative stress and augmented insulin signaling in the liver and skeletal muscle of Akita mice and in rat skeletal myoblast cell lines, suggesting that ORP150 improves the insulin sensitivity impaired by oxidative stress.

## RESEARCH DESIGN AND METHODS

For Western blotting, anti-human ORP150 (1  $\mu$ g/ml) (9), anti-insulin receptor substrate (IRS)-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine (Calbiochem-Novabiochem, San Diego, CA), and anti- $\beta$ -actin Ab IgG (Sigma Chemical, St. Louis, MO) were used. We used anti-human ORP150 IgG (5  $\mu$ g/ml) (16) and anti-insulin IgG for immunohistochemical analysis. The construct of the rat insulin promoter was a kind gift from Dr. Richard D.

Palmiter at the University of Washington. L6 cells were purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank (no. IFO50364; Osaka, Japan).

All procedures involving animals were approved by the Animal Care and Use Committee of Kanazawa University. ORP150 transgenic mice using a cytomegalovirus immediate early enhancer-chicken  $\beta$ -actin hybrid (CAG) promoter (16) were a kind gift from the HSP institute (17). ORP150 heterozygous knockout mice have been generated previously (18). Akita mice established from C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Transgenic mice using insulin promoter were generated at the Genome Information Research Center (Osaka University, Osaka, Japan) (10). The genotype of the mutant mice was determined by Southern blot analysis and PCR. These mice were crossed into the C57BL/6 background.

The levels of HbA<sub>1c</sub> were measured from tail-vein blood using a DCA2000 analyzer (Bayer Medical, Tokyo, Japan) (19). The concentration of insulin was measured using an insulin enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's instructions (Shibayagi, Shibukawa, Japan). **Glucose metabolism of mutant mice.** Intraperitoneal glucose tolerance tests (IPGTTs) and insulin tolerance tests (ITTs) were performed as described previously (20). Hyperinsulinemic-euglycemic clamp was performed as described previously (21,22). The rates of glucose appearance ( $R_a$ ) and disappearance ( $R_d$ ) were calculated according to Steele's non-steady-state equations. Endogenous glucose production (EGP) was calculated as the difference between the tracer-derived  $R_a$  and exogenous infusion rates of glucose (GIRs) and tracer.

**Assessment of insulin signaling.** Animals that fasted overnight were anesthetized and injected with either saline or 5 IU human insulin via the inferior vena cava. The liver was obtained after 2 min and the skeletal muscle after 5 min. L6 cells were harvested after 5 min treatment with hydrogen peroxide. Protein samples from the liver, skeletal muscle, and L6 cells underwent immunoprecipitation for IRS-1 followed by Western blotting with an anti-IRS-1 antibody and anti-phosphorylated IRS-1 antibody.

**Treatment with  $\alpha$ -lipoic acid.** At 6 weeks of age, C57BL/6 and Akita mice received an intraperitoneal injection of either vehicle or 30 mg/kg body wt  $\alpha$ -lipoic acid over 5 days (23).

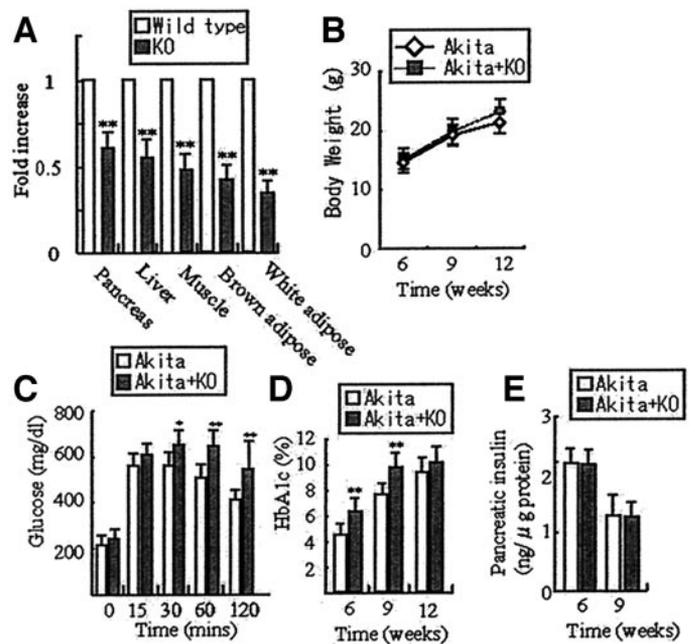
**Protein oxidation detection.** The formation of protein carbonyl groups was assessed by a OxyBlot protein oxidation detection kit (Integen) used in accordance with the manufacturer's protocol.

**Adenovirus infection.** Adenovirus coding for ORP150 in the sense (Ad/S-ORP150) or antisense (Ad/AS-ORP150) orientation and coding LacZ (AxCALacZ) have been generated previously (10). L6 cells were infected with an adenovirus at 100 multiplicities of infection (MOI) for 24 h as described previously.

**Data analysis.** Statistical analysis was performed by either an unpaired *t* test or an ANOVA followed by multiple comparison analysis using Newman-Kuehl's equation. Where indicated, the data were analyzed by a two-way ANOVA followed by multiple contrast analysis. For nonparametric data, either a Kruskal-Wallis analysis or  $\chi^2$  analysis was applied.

**RESULTS**

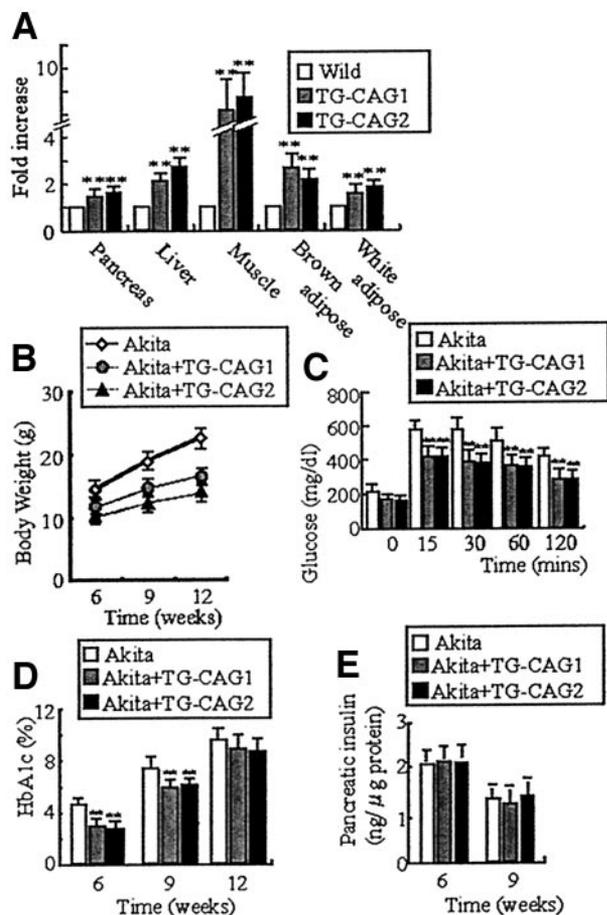
**Targeted disruption of ORP150 heterozygously accelerated the onset of diabetes in Akita mice.** ORP150 heterozygous mice (ORP150<sup>-/+</sup>) were mated with heterozygous Akita mice (Ins2<sup>WT/C96Y</sup>). Consistent with a previous report (16), ORP150 levels of ORP150<sup>-/+</sup> mice were reduced by ~50% compared with those of their wild-type littermates (Fig. 1A), and similar results were obtained between ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> and Ins2<sup>WT/C96Y</sup> mice (data not shown). There was no significant difference in the body weights of ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> and Ins2<sup>WT/C96Y</sup> mice (Fig. 1B). The IPGTT showed there was no significant difference between ORP150<sup>-/+</sup> and their wild-type littermates (data not shown), whereas at 6 weeks the serum glucose levels of ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> were significantly higher than those of Ins2<sup>WT/C96Y</sup> mice (Fig. 1C). The measurement of HbA<sub>1c</sub> showed a similar trend as IPGTT at 6 and 9 weeks; however, at 12 weeks there was no significant difference in HbA<sub>1c</sub> levels between Ins2<sup>WT/C96Y</sup> and ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> mice (Fig. 1D). Contrary to our prediction, there was no significant difference in the insulin content of the pancreas of ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> and Ins2<sup>WT/C96Y</sup> mice (Fig. 1E).



**FIG. 1.** Glucose and insulin homeostasis in Akita mice heterozygous for ORP150 deficiency. **A:** Indicated tissues of ORP150<sup>-/+</sup> and wild-type littermates underwent immunoblotting, and semiquantitative analysis was performed (*n* = 4). **B:** Growth curve of Ins2<sup>WT/C96Y</sup> and ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> mice (*n* = 8). **C:** The glucose tolerance test was performed on male Ins2<sup>WT/C96Y</sup> and ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> mice (*n* = 5). **D:** Blood HbA<sub>1c</sub> levels measured in Ins2<sup>WT/C96Y</sup> and ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> mice (*n* = 6). **E:** Pancreatic insulin content of Ins2<sup>WT/C96Y</sup> and ORP150<sup>-/+</sup>Ins2<sup>WT/C96Y</sup> mice (*n* = 5). Data are means  $\pm$  SD. \**P* < 0.05 and \*\**P* < 0.01 by multiple comparison analysis compared with the control.

**Glucose metabolism of Akita mice overexpressing ORP150.** ORP150 transgenic mice generated using the CAG promoter (ORP150<sup>CAG</sup>) were mated with Ins2<sup>WT/C96Y</sup> mice. The levels of ORP150 protein were significantly greater in ORP150<sup>CAG</sup> compared with their wild-type littermates (Fig. 2A), and similar results were obtained between ORP150<sup>CAG</sup>Ins2<sup>WT/C96Y</sup> and Ins2<sup>WT/C96Y</sup> (data not shown). Consistent with a previous report (17), ORP150<sup>CAG</sup>Ins2<sup>WT/C96Y</sup> had significantly lower body weights than Ins2<sup>WT/C96Y</sup> mice (Fig. 2B). IPGTT showed that at 6 weeks, the glucose tolerance of ORP150<sup>CAG</sup>Ins2<sup>WT/C96Y</sup> was greater than that of Ins2<sup>WT/C96Y</sup> (Fig. 2C). HbA<sub>1c</sub> showed a similar trend as IPGTT at 6 and 9 weeks; however, at 12 weeks there was no significant difference (Fig. 2D). There was no significant difference in the insulin content of the pancreas (Fig. 2E).

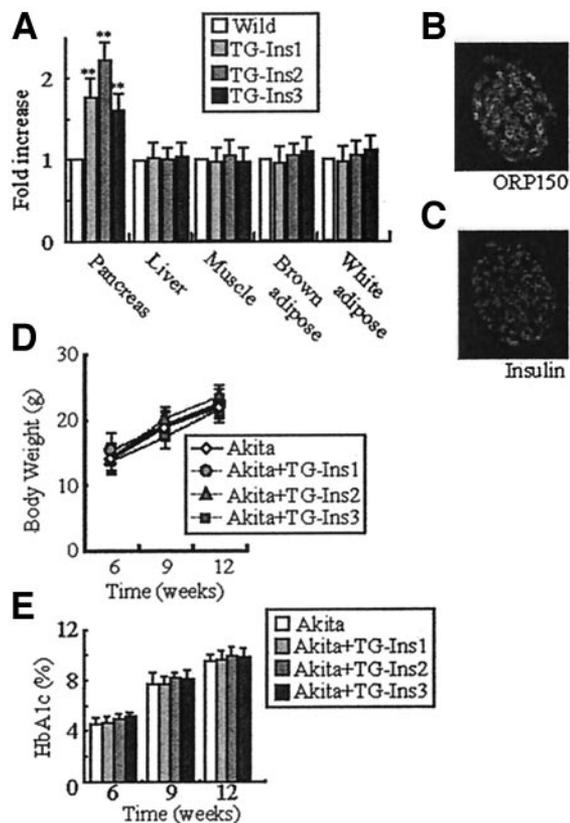
To determine whether overexpression of ORP150 in  $\beta$ -cells of the islets improves the glucose intolerance of Ins2<sup>WT/C96Y</sup>, ORP150 transgenic mice were generated using the rat insulin promoter (ORP150<sup>Ins</sup>), as described in RESEARCH DESIGN AND METHODS. Western blot analysis revealed that ORP150 levels in the pancreas of ORP150<sup>Ins</sup> mice lines were significantly higher than those of their nontransgenic littermates. In comparison, there was no significant difference in the levels of ORP150 in the liver, skeletal muscle, white fat tissue, and brown fat tissue between the three lines of ORP150<sup>Ins</sup> and their wild-type littermates (Fig. 3A). Furthermore, immunohistochemical analysis showed that ORP150 expression in the pancreas of ORP150<sup>Ins</sup> was limited to the islets of Langerhans and overlapped with that of insulin (Fig. 3B and C), suggesting that, as pre-



**FIG. 2.** Glucose and insulin homeostasis in Akita mice overexpressing ORP150 using CAG promoter. **A:** Indicated tissues of two lines of ORP150<sup>CAG</sup>(TG-CAG1 and 2) and their wild-type littermates (wild) underwent immunoblotting, and semiquantitative analysis was performed ( $n = 4$ ). **B:** The growth curve of Ins2<sup>WT/CY</sup> (Akita) and ORP150<sup>CAG</sup>Ins2<sup>WT/CY</sup> (Akita+TG-CAG1 and -2) ( $n = 8$ ). **C:** Male Ins2<sup>WT/CY</sup> and ORP150<sup>CAG</sup>Ins2<sup>WT/CY</sup> underwent the glucose tolerance test ( $n = 5$ ). **D:** Blood HbA<sub>1c</sub> levels measured in Ins2<sup>WT/CY</sup> and ORP150<sup>CAG</sup>Ins2<sup>WT/CY</sup> ( $n = 6$ ). **E:** Pancreatic insulin content of Ins2<sup>WT/CY</sup> and ORP150<sup>CAG</sup>Ins2<sup>WT/CY</sup> mice ( $n = 5$ ). Data are means  $\pm$  SD.  $**P < 0.01$  by multiple comparison analysis compared with the control.

dicted, the transgene was expressed in  $\beta$ -cells. There was no significant difference between the growth curves of Ins2<sup>WT/C96Y</sup> and ORP150<sup>Ins</sup>Ins2<sup>WT/C96Y</sup> mice (Fig. 3D). Both glucose tolerance assessed by IPGTT (data not shown) and measurement of HbA<sub>1c</sub> (Fig. 3E) were not significantly different between Ins2<sup>WT/C96Y</sup> and ORP150<sup>Ins</sup>Ins2<sup>WT/C96Y</sup> mice. In addition, there was no significant difference in the pancreatic levels of insulin in Ins2<sup>WT/C96Y</sup> and ORP150<sup>Ins</sup>Ins2<sup>WT/C96Y</sup> mice (data not shown). The results shown in Figs. 1 and 2 suggest that ORP150 improves the glucose intolerance of Akita mice but not the secretion of insulin.

**ORP150 is involved in insulin sensitivity.** To determine whether ORP150 expression was lessened by fasting (14), the pancreas (Fig. 4A), liver (Fig. 4B), and skeletal muscle (Fig. 4C) of C57BL/6 starved for indicated times were used for Western blotting with an anti-ORP150 antibody. In contrast to previous findings (14), there was no significant difference in the levels of ORP150 in the pancreatic tissue of control and starved mice, whereas star-

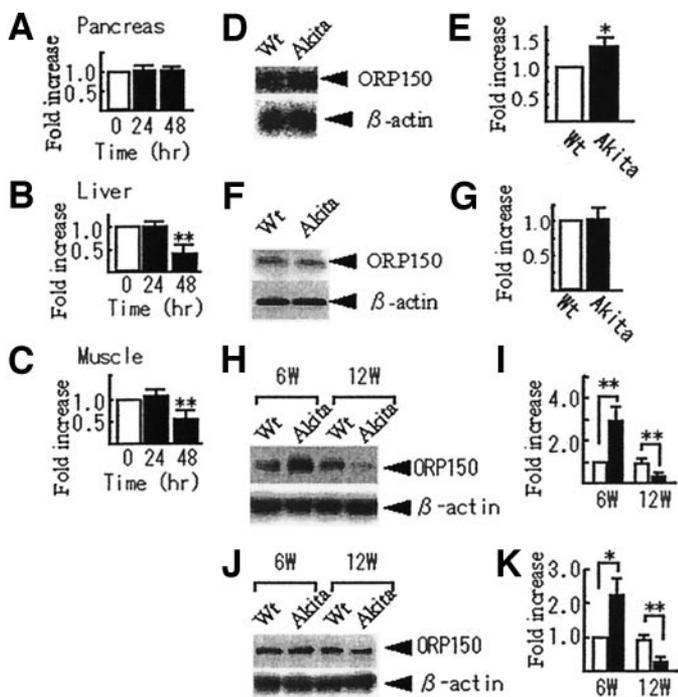


**FIG. 3.** Glucose and insulin homeostasis in Akita mice overexpressing ORP150 using insulin promoter. **A:** Indicated tissues of two lines of three lines of ORP150<sup>Ins</sup> (TG-ins1, -2, and -3) and their wild-type littermates (wild) underwent immunoblotting, and semiquantitative analysis was performed ( $n = 4$ ). **B** and **C:** Pancreatic sections of ORP150<sup>Ins</sup> mice that underwent immunohistochemical analysis using an anti-ORP150 (**B**) and anti-insulin antibody (**C**). **D:** The growth curve of Ins2<sup>WT/CY</sup> (Akita) and ORP150<sup>Ins</sup>Ins2<sup>WT/CY</sup> (Akita+ TG-ins1, -2, and -3) ( $n = 8$ ). **E:** Blood HbA<sub>1c</sub> levels measured in Ins2<sup>WT/CY</sup> and ORP150<sup>Ins</sup>Ins2<sup>WT/CY</sup> mice ( $n = 6$ ). Data are means  $\pm$  SD ( $n = 4$ ).  $**P < 0.01$  by multiple comparison analysis compared with the control.

vation for 48 h significantly reduced the levels of ORP150 in the liver and skeletal muscle.

We assessed the levels of ORP150 in Akita mice. The levels of ORP150 transcript in the pancreas of Ins2<sup>WT/C96Y</sup> were significantly higher than those of C57BL/6 (Fig. 4D and 4e). However, there was no significant difference in the levels of ORP150 protein between Ins2<sup>WT/C96Y</sup> and C57BL/6 mice (Fig. 4F and G). Immunoblotting also revealed that the levels of ORP150 in the liver (Fig. 4H and I) and skeletal muscle (Fig. 4J and K) of Ins2<sup>WT/C96Y</sup> were significantly greater compared with that of C57BL/6 mice at 6 weeks, whereas those of Ins2<sup>WT/C96Y</sup> were significantly lower compared with those of C57BL/6 mice at 12 weeks.

To determine whether ORP150 is involved in the insulin sensitivity of Ins2<sup>WT/C96Y</sup>, an ITT was performed. The ITT revealed that overexpression of ORP150 increased the insulin sensitivity of Ins2<sup>WT/C96Y</sup> (Fig. 5A), whereas heterozygous disruption of ORP150 reduced insulin sensitivity (Fig. 5B). To further investigate these findings, ORP150<sup>CAG</sup> and ORP150<sup>-/+</sup> mice underwent hyperinsulinemic-euglycemic clamp. As described previously (17), ORP150<sup>CAG</sup> mice displayed the phenotype of myocardial degeneration and died of heart failure during the clamp test. The average GIR of 90–120 min in ORP150<sup>-/+</sup> was significantly lower than that of their littermates (Fig. 5C).



**FIG. 4.** Expression of ORP150 in starved and diabetic mice. *A–C*: Wild-type mice (C57BL/6) were starved 0–48 h and the indicated tissues harvested for Western blot analysis using an anti-ORP150 and anti- $\beta$ -actin antibody; semiquantitative analysis is shown. The intensity of ORP150 is represented as the percentage increase over the control. *D* and *E*: Total RNA from the pancreas of male wild-type (C57BL/6) and 6-week-old Akita mice used in Northern blot analysis. A typical example of the blot is shown in *D*. Semiquantitative analysis is shown in *E*, where the intensity of ORP150 is represented as the percentage increase over the control. *F–K*: Protein samples from the pancreas (*F* and *G*), liver (*H* and *I*), and skeletal muscle (*J* and *K*) of male C57BL/6 and Akita mice (*Ins2*<sup>WT/C96Y</sup>) used in Western blot analysis. A typical example of the blot is shown in *F*, *H*, and *J*. Semiquantitative analysis is shown in *G*, *I*, and *K*, where the intensity of ORP150 is represented as the percentage increase over the control. Data are shown as means  $\pm$  SD ( $n = 4$ ). \* $P < 0.05$  and \*\* $P < 0.01$  by multiple comparison analysis compared with the control.

EGP showed there was no significant difference between ORP150<sup>-/+</sup> and their wild-type littermates (data not shown). We assessed insulin signaling in *Ins2*<sup>WT/C96Y</sup>, ORP150<sup>CAG</sup> *Ins2*<sup>WT/C96Y</sup>, and ORP150<sup>-/+</sup> *Ins2*<sup>WT/C96Y</sup> mice. After overnight starvation and treatment with either saline or insulin, protein was extracted from the liver (Fig. 5*D* and *E*) and skeletal muscle (Fig. 4*F* and *G*) for immunoprecipitation with an anti-IRS-1 IgG, followed by Western blot analysis with an anti-phosphorylated IRS-1 IgG and anti-IRS-1. The levels of phosphorylated IRS-1 in ORP150<sup>CAG</sup> *Ins2*<sup>WT/C96Y</sup> were increased compared with *Ins2*<sup>WT/C96Y</sup> but were reduced in ORP150<sup>-/+</sup> *Ins2*<sup>WT/C96Y</sup> mice (Fig. 4*E* and *G*). These results indicate that ORP150 might play a role in the insulin sensitivity of the liver and skeletal muscle but not in the secretion of insulin.

**ORP150 decreased oxidative stress in Akita mice.** We speculated that oxidative stress caused by hyperglycemia might induce ORP150 expression. To determine whether there was a relationship between oxidative stress and the induction of ORP150 in diabetes, Akita mice at 5 weeks were treated with  $\alpha$ -lipoic acid (ALA), an antioxidant drug that improves insulin resistance (23). Northern blot analysis showed that ALA reduced the levels of ORP150 transcript in the liver (Fig. 6*A* and *B*) and skeletal muscle (Fig. 6*C* and *D*) of Akita mice. In addition, 10–40  $\mu$ mol/l

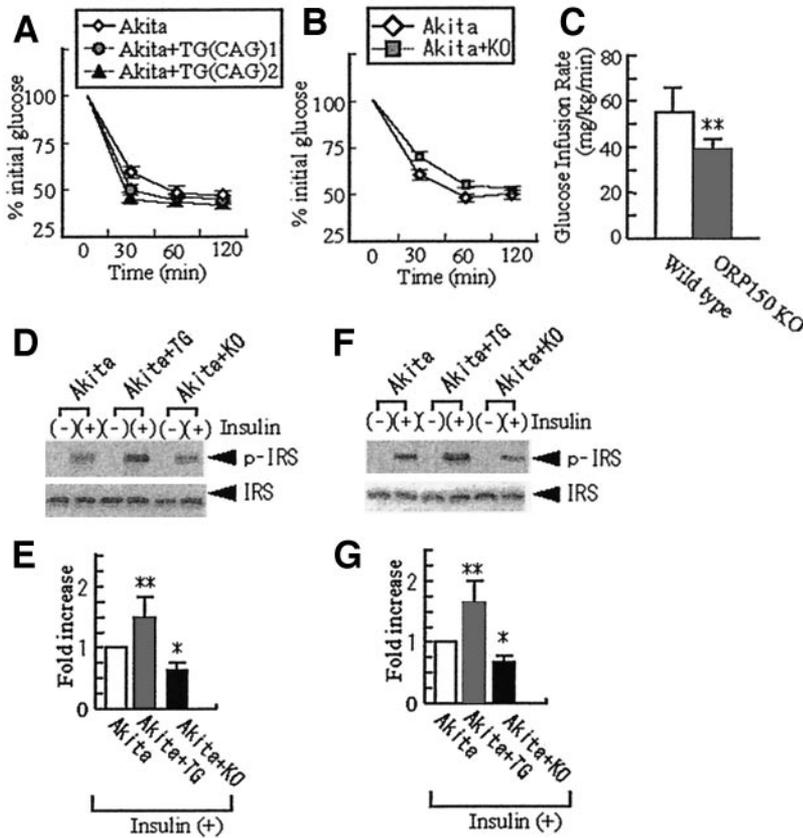
hydrogen peroxide induced ORP150 and GRP78 transcripts in L6 skeletal myoblast cells (Fig. 7*A* and *B*). In contrast, Chop transcripts were only induced by 100  $\mu$ mol/l hydrogen peroxide (Fig. 7*A* and *C*). ORP150 and GRP78 transcript expression peaked 6–12 h after the treatment with hydrogen peroxide (data not shown). Immunoblot analysis showed the similar results as Northern blot analysis (data not shown). These data suggest that oxidative stress could induce ORP150 and GRP78. To further investigate the role of hydrogen peroxide in ER, L6 cells were treated with hydrogen peroxide for 24 h and then L6 cells were treated with 20  $\mu$ mol/l hydrogen peroxide and tunicamycin (TM). TM induced ORP150 (Fig. 7*D* and *E*) and GRP78 (Fig. 7*D* and *F*), and hydrogen peroxide decreased their induction (Fig. 7*D–F*). These data are consistent with the in vivo data that levels of ORP150 in liver and muscle of Akita mice were decreased compared with those of wild-type mice at 12 weeks. Taken together, these data suggest that oxidative stress could induce ER chaperone; however, prolonged oxidative stress attenuates the induction of ER chaperone.

Furthermore, Western blotting using an anti-DNP (1-3 dinitrophenyl hydrazine) antibody revealed that overexpression of ORP150 reduced the levels of carbonylated protein in Akita mice (Fig. 8*A*) but that carbonylated protein was increased with heterozygous disruption of ORP150 (Fig. 8*B*). To further determine a possible role for ORP150, the levels of ORP150 were modulated in L6 myoblast cells using adenoviruses coding for ORP150 in the sense (Ad/S-ORP150) or antisense (Ad/AS-ORP150) orientation. Treatment of L6 cells with Ad/S-ORP150 significantly increased the levels of ORP150, whereas treatment with Ad/AS-ORP150 suppressed ORP150 levels (Fig. 8*C*). L6 cells infected with Ad/S-ORP150, Ad/AS-ORP150, or an adenovirus encoding LacZ (AxCALacZ) were treated with 20  $\mu$ mol/l hydrogen peroxide and then used in Western blot analysis in conjunction with an anti-DNP antibody. The levels of oxidative protein in L6 cells treated with hydrogen peroxide were suppressed Ad/S-ORP150 but increased by Ad/AS-ORP150 (Fig. 8*D*). Using immunoprecipitation, insulin signals were further assessed in L6 cells treated with hydrogen peroxide. Ad/S-ORP150 consistently enhanced phosphorylation of IRS-1, but phosphorylation was suppressed by Ad/AS-ORP150 (Fig. 8*E* and *F*), suggesting that the ER chaperone might protect insulin signaling from oxidative stress in myoblast cells.

**DISCUSSION**

In this report, we demonstrated that ORP150 expression could be induced by hydrogen peroxide in myoblast cells and that overexpression of ORP150 using an adenovirus reduced oxidized protein. However, in a previous study, we showed that ORP150 had no influence on hydrogen peroxide-induced cell death (9,10). This discrepancy might arise from the different concentrations of hydrogen peroxide added to the cells. In previous reports, 100  $\mu$ mol/l to 10 mmol/l hydrogen peroxide has been added to either 293 cells or primary neurons (9,10), whereas in this study, 10–40  $\mu$ mol/l hydrogen peroxide induced ORP150 but produced no significant difference in cell growth and death (data not shown).

ORP150<sup>CAG</sup> *Ins2*<sup>WT/C96Y</sup> showed lower body weight com-



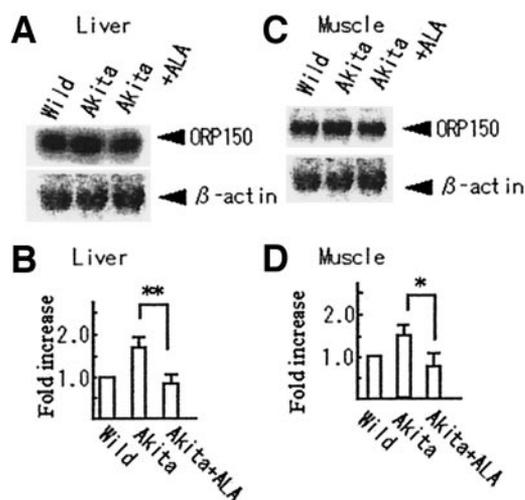
**FIG. 5.** Assessment of insulin sensitivity of mutant mice. *A* and *B*: ITT of  $Ins2^{WT/CY}$ ,  $ORP150^{CAG}Ins2^{WT/CY}$ , and  $ORP150^{-/-}Ins2^{WT/CY}$  mice ( $n = 5$ ). *C*: GIR of wild-type littermates and  $ORP150^{-/-}$  mice (seven littermates and five  $ORP150^{-/-}$ ). *D–G*: Phosphorylation of IRS-1 in  $Ins2^{WT/CY}$ ,  $ORP150^{CAG}Ins2^{WT/CY}$ , and  $ORP150^{-/-}Ins2^{WT/CY}$  mice were assessed. A typical example of the blot is shown in *D* and *F*. Semiquantitative analysis is shown in *E* and *G* ( $n = 4$ ). Data are means  $\pm$  SD ( $n = 4$ ). \* $P < 0.05$  and \*\* $P < 0.01$  by multiple comparison analysis compared with the control.

pared with the nontransgenic Akita ( $Ins2^{WT/C96Y}$ ). It is possible that these differences in body weight might account for the differences in insulin sensitivity. To eliminate this possibility, we performed IPGTT and ITT using  $ORP150^{CAG}$  and weight-matched control. IPGTT showed no significant difference between  $ORP150^{CAG}$  and weight-matched control (see online appendix at <http://diabetes.>

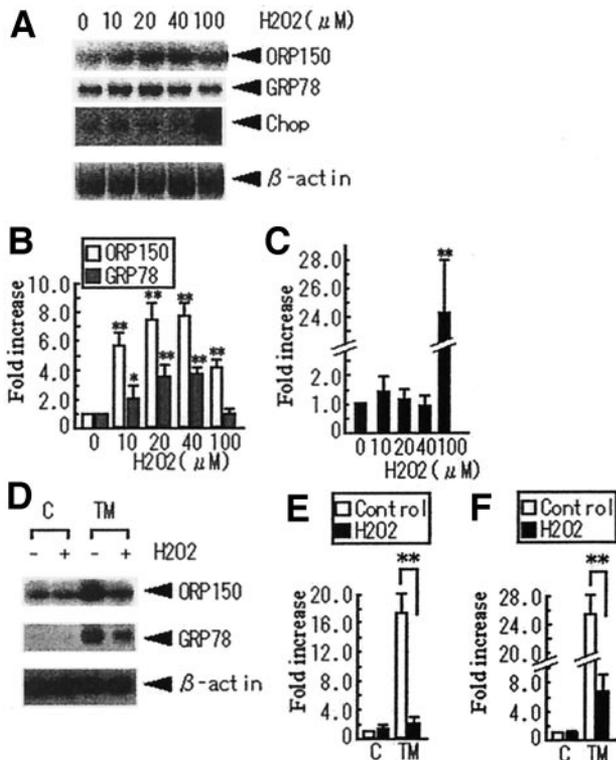
[diabetesjournals.org](http://diabetesjournals.org)). However, ITT showed that overexpression of ORP150 improved insulin sensitivity compared with weight-matched control (online appendix).

Using immunoblotting, we have shown that fasting reduces the levels of ORP150 in the liver and skeletal muscle (Fig. 4*B* and *C*). These data are consistent with a previous report showing that the expression of some ER chaperones, including ORP150, was lower in the liver of mice fed energy-restricted food compared with that of freely fed mice (24). In addition, we have shown that the levels of ORP150 in the liver and skeletal muscle of Akita mice were significantly greater compared with that of wild-type mice (Fig. 4*H–K*). Given that mutation of the insulin two gene is responsible for the phenotype of Akita mice, we expected hyperglycemia might secondarily induce ORP150 expression in the liver and skeletal muscle. This idea is consistent with our pilot data showing increased ORP150 expression in the liver and skeletal muscle of *db/db* diabetic mice or C57BL/6 mice treated with streptozotocin.

Although there is no significant difference in glucose levels during IPGTT between  $ORP150^{-/+}$  and wild-type littermates (data not shown), hyperinsulinemic-euglycemic clamp revealed that insulin sensitivity of  $ORP150^{-/+}$  was greater than that of wild-type littermates (Fig. 5*C*). As described in RESEARCH DESIGN AND METHODS, excess insulin was infused to maintain levels of glucose during clamp test, and insulin accelerates the process of mRNA translation (25) and results in increasing protein synthesis and ER stress. These reports led us to the idea that more ORP150 is required during clamp test than during IPGTT, and this is consistent with our pilot study that ORP150



**FIG. 6.** Antioxidant drug decreased the expression of ORP150. C57BL/6 and Akita mice were treated with either saline or ALA, and the indicated tissues underwent Northern blot analysis for ORP150 and  $\beta$ -actin. A typical example of the blot is shown in *A* and *C*. Semiquantitative analysis is shown in *B* and *D*, where the intensity of ORP150 is represented as the percentage increase over the levels of  $\beta$ -actin. Data are means  $\pm$  SD ( $n = 4$ ). \* $P < 0.05$  and \*\* $P < 0.01$  by multiple comparison analysis compared with the control.

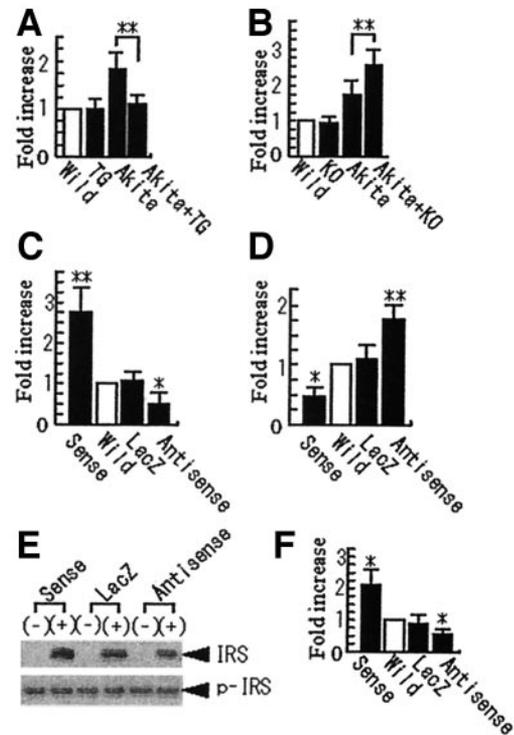


**FIG. 7.** The relationship between ORP150 and oxidative stress. *A–C:* L6 myoblast cells treated with hydrogen peroxide underwent Northern blot analysis for ORP150, GRP78, Chop, and  $\beta$ -actin. A typical example of the blot is shown in *A*. Semiquantitative analysis is shown in *B* and *C*, where the intensity of ORP150 (*B*), GRP78 (*B*), and Chop (*C*) is represented as the percentage increase over the control (0 h) normalized to the levels of  $\beta$ -actin. Data are means  $\pm$  SD ( $n = 4$ ). *D–F:* L6 myoblast cells, treated with hydrogen peroxide for 24 h, were subjected to TM and hydrogen peroxide for 6 h and then underwent Northern blot analysis for ORP150, GRP78, and  $\beta$ -actin. A typical example of the blot is shown in *D*. Semiquantitative analysis is shown in *E* and *F*, where the intensity of ORP150 (*E*) and GRP78 (*F*) is represented as the percentage increase over the control (0 h) normalized to the levels of  $\beta$ -actin ( $n = 4$ ). Data are means  $\pm$  SD. \* $P < 0.05$  and \*\* $P < 0.01$  by multiple comparison analysis compared with the control.

levels in liver and muscle of *db/db* mice were greater than that of Akita mice (data not shown).

We have reported that ORP150 enhances the secretion of VEGF in wound healing and tumor formation (11,12), and, therefore, we expected that ORP150 might enhance the secretion of ACRP30/adiponectin, the expression of which correlates with insulin sensitivity (26), resulting in improved insulin resistance of Akita mice. To determine whether ORP150 increases the secretion of ACRP30, we assessed the levels of ACRP30 in serum and white/brown adipose tissues of *Ins2*<sup>WT/C96Y</sup>, *ORP150*<sup>-/+</sup>*Ins2*<sup>WT/C96Y</sup>, and *ORP150*<sup>CAG</sup>*Ins2*<sup>WT/C96Y</sup> by ELISA; however, no significant differences were shown between *Ins2*<sup>WT/C96Y</sup> and *ORP150*<sup>-/+</sup>*Ins2*<sup>WT/C96Y</sup> or between *Ins2*<sup>WT/C96Y</sup> and *ORP150*<sup>CAG</sup>*Ins2*<sup>WT/C96Y</sup> (online appendix).

In contrast, the ER of proteins consumes oxidizing equivalents during the process of disulphide-bond formation (27,28), and ER stress in *PERK*<sup>-/-</sup> or *ATF4*<sup>-/-</sup> cells leads to the acute production of reactive oxygen species (ROS) through the accumulation of proteins oxidized by protein disulfide isomerase (29). These findings suggest that ER can reduce ROS in the liver and skeletal muscle, as well as improve insulin sensitivity in type 2 diabetes. As a



**FIG. 8.** ORP150 enhanced insulin signaling. *A* and *B:* The liver and skeletal muscle of wild-type (wild), *ORP150*<sup>-/+</sup> (KO), *ORP150*<sup>CAG</sup> (TG), *Ins2*<sup>WT/C96Y</sup> (Akita), *ORP150*<sup>-/+</sup>*Ins2*<sup>WT/C96Y</sup> (Akita+KO), and *ORP150*<sup>CAG</sup>*Ins2*<sup>WT/C96Y</sup> (Akita+TG) mice that underwent Western blot analysis using an anti-DNP antibody and semiquantitative analysis ( $n = 4$ ). *C* and *D:* L6 cells infected by Ad/S-ORP150, Ad/AS-ORP150, or AxCALacZ and treated with hydrogen peroxide underwent Western blot analysis using either an anti-ORP150 antibody or anti-DNP antibody. Semiquantitative analysis is shown, where the intensity of ORP150 (*C*) and anti-DNP antibody (*D*) is represented as the percentage increase over the control (not infected with adenovirus) normalized to the levels of  $\beta$ -actin ( $n = 4$ ). *E* and *F:* L6 cells infected by Ad/S-ORP150, Ad/AS-ORP150, or AxCALacZ and treated with hydrogen peroxide were stimulated with either saline (-) or 100 nmol/l of human insulin (+) underwent immunoprecipitation of IRS-1 followed by Western blotting with an anti-phosphotyrosine antibody and anti-IRS-1 antibody. A semiquantitative analysis was performed ( $n = 4$ ). Data are means  $\pm$  SD. \* $P < 0.05$  and \*\* $P < 0.01$  by multiple comparison analysis compared with the control.

molecular chaperone, ORP150 increases folding capacity and enhances protein secretion during ER stress (10,11,12,30). Consequently, ORP150 could enhance the role of the ER by reducing excess oxidizing equivalents and improving insulin sensitivity in type 2 diabetes.

Taken together, these data demonstrate that the ER chaperone ORP150 could remit insulin resistance caused by hyperglycemia by reducing oxidative stress and that it might be a novel therapeutic target to reduce the insulin resistance characteristic of type 2 diabetes.

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